UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS

Development of new antibiotics against Bacillus anthracis

"Documento Definitivo"

Doutoramento em Química
Especialidade de Química Orgânica

João Pedro Almeida Pais

Tese orientada por:
Amélia Pilar Grases Santos Silva Rauter
Ricardo Pedro Moreira Dias
Teresa Paulo Tavares da Silva Alves

Documento especialmente elaborado para a obtenção do grau de doutor

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Trabalho financiado pela Fundação para a Ciência e Tecnologia e CIPAN através da bolsa SFRH/BDE/51957/2012.

2018

Abstract

The rise of bacterial resistance has driven the search for new antimicrobials employing not only innovative mechanisms of action, but also new characteristics such as high selectivity or multitargeting capabilities, whilst the search for new biological targets continues.

Past work demonstrated the potential of carbohydrate chemistry, with a new family of surface-active deoxy sugars showing promising antimicrobial activity and surprising selectivity towards the *Bacillus* genus. These characteristics, and the absence of correlation between MIC and CMC values for the most active compound, motivated the creation of a small library of compounds with the optimal structural features for these alkyl deoxy glycosides. Simultaneously, the referred antimicrobial and surfactant characteristics showed remarkable differences when compared to the literature of similar alkyl glycosides, hence the study of a possible mechanism of action, independent of the expected surfactant effect was undertaken.

The synthesis of antimicrobial derivatives was target oriented, and the methodology applied successfully provided the desired analogs in good yields. The evaluation of antimicrobial activity allowed to establish the essential structural features of this alkyl deoxy glycoside family, leading to dodecyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside (**1**) as the ideal compound for the biological studies. This compound showed to be ineffective in the disinfection of surfaces and lack sporicidal activity, however, bacterial mutants resistant to the main families of antibiotics revealed unaltered susceptibility (MIC=16 μg/mL), and no resistance development was observed after continuous exposure.

The study of the mode of action was initiated by kinetic characterization of the bactericidal action, followed by potential target assessment by measuring the differential metabolic response when exposed to compound **1**, defining the main metabolic pathways affected. By establishing a hierarchical relationship model between them, the most probable targets were identified: ABC and PTS transport systems and the amino acyl t-RNA biosynthesis. The creation of mutant libraries helped to understand that the

existence of a specific molecular target is very unlikely, since no significant alteration of the susceptibility of all mutant libraries to compound **1** was observed.

Hence, the importance of the bacterial ultrastructures to the antibacterial activity was assessed and the susceptibility of protoplasts and spheroplasts revealed that, for Gram-negative bacteria, the ultrastructures enveloping the cytoplasmic membrane are responsible for the resistance to this compound, while in Gram-positive bacteria they have no effect. This also means that the specificity observed to *Bacillus* spp., here exemplified *S. aureus*, results from the characteristics of the bacterial membrane. The impact of compound **1** in the cellular envelope was achieved using AFM techniques, unequivocally demonstrating that these compounds cause alterations of cell surface morphology and cellular damage, even at sub inhibitory concentrations.

Additionally, an opportunity emerged to apply this expertise in carbohydrate synthesis to develop new tools for early Alzheimer's disease diagnosis and possible therapeutics, aiming to improve solubility and bioavailability of known bioactive molecules while providing additional selectivity. A simple, low-cost synthetic strategy was paramount to the challenge proposed: the glucoconjugation of fluorescein, quinolone, curcumin and cyclohexylpiperazine derivatives. The methodologies chosen resulted in the successful glucosilation, particularly for the latter.

In summary, the multidisciplinary work here reported denotes the capabilities and importance of carbohydrate chemistry for all life sciences. Allowed to successfully establish a correlation between the structure and the bioactivity of alkyl deoxy glycosides, fully characterized the exhibited bactericidal action and provided essential information regarding the peculiarities of the mechanism of action, to be used as guidelines for future work. Moreover, it demonstrated potential in assisting the development of new tools against AD.

Keywords: multidisciplinary, surfactant glycosides, glycosylation, antibacterial, mode of action

Resumo alargado

O aumento da resistência bacteriana e o consequente declínio na eficácia dos antibióticos disponíveis têm impulsionado o desenvolvimento de novos antimicrobianos com mecanismos de ação inovadores. Esta realidade representa um desafio ao nível das características antibacterianas pretendidas, como a alta seletividade e a capacidade de atuação, simultânea, em múltiplos alvos, paralelamente à contínua procura por novos alvos biológicos.

Trabalhos preliminares permitiram demonstrar o potencial da química dos carbohidratos na resposta a essas necessidades, tendo sido criada uma nova família de desoxi glicósidos surfactantes, que apresentam atividade antimicrobiana promissora e seletividade para as bactérias do género *Bacillus*. Estas características, bem como a ausência de correlação entre os valores de CIM e CMC para o composto mais ativo, motivaram a criação de uma biblioteca de compostos para determinar a estrutura otimizada desta família de antimicrobianos. Simultaneamente, a bioatividade demonstrada e as propriedades surfactantes evidenciaram diferenças notáveis quando comparadas com os alquil glicósidos semelhantes, encontrados na literatura. Tal facto motivou o estudo de um possível mecanismo de ação para estes compostos, independente do efeito surfactante esperado.

Para além disso, procedemos à aplicação da síntese de carbohidratos no desenvolvimento de novas ferramentas para o diagnóstico precoce da doença de Alzheimer e para possíveis terapias, com o intuito de melhorar a solubilidade e biodisponibilidade de moléculas bioativas. Neste contexto, o presente trabalho teve como foco a glicoconjugação de estruturas moleculares quelantes de cobre e moléculas indutoras de P-gp.

As estratégias de glicosilação aplicadas tanto na criação de novos antimicrobianos, como na melhoria de moléculas bioativas, foram adaptadas às suas especificidades. Assim, a glicoconjugação de derivados de fluoresceína, quinolona, curcumina e ciclohexilpiperazina, exigiu uma abordagem exploratória. Para além disso, a adoção de uma estratégia sintética simples e de baixo custo foi requisito à elaboração deste trabalho, pelo que recorremos ao pentaacetato de glucose como doador de glicosilo.

Porém, apurou-se que o método de glucoconjugação com trifluoreto de boro como catalisador, produziu rendimentos muito baixos (<10%) para todos os compostos. Para tornar o sistema mais reativo, o pentaacetato de glucose foi quantitativamente convertido no correspondente brometo de glucosilo, e aplicado numa metodologia de transferência de fase conduziu a rendimentos mais elevados, particularmente no caso do derivado de ciclohexilpiperazina (η=47%). No entanto, mesmo após terem sido reunidos esforços no sentido da otimização deste sistema reacional, não foi possível obter glucósidos de curcumina.

A abordagem sintética para o desenvolvimento de derivados antimicrobianos focou-se na síntese de alquil 2-desoxi-glicósidos, com preferencial obtenção dos anómeros α, sendo estes os mais ativos. Diante disso e com base em trabalhos anteriormente desenvolvidos, a síntese de tridecil, dodecil, undecil, decil, 1,1-H,H-perfluorodecil e 2-octildodecil 2,6-didesoxi-L-arabino-hexopiranósidos foi realizada pela reação de peracetil L-ramnal (obtido a partir de L-ramnose em três etapas), com os álcoois correspondentes. Esta glicosilação foi catalisada por bromidrato de trifenilfosfano e subsequente desproteção dos produtos obtidos. Tendo sido conseguida a otimização da metodologia para um procedimento one-pot, esta permitiu-nos obter melhores rendimentos dos produtos sintetizados em, respetivamente, 69%, 67%, 56%, 71% e 59%.

Adicionalmente, foi sintetizado o composto dodecil 2,6-didesoxi-2-iodo-α-L-arabino-hexopiranósido (η=61%), através da utilização de NIS como catalisador para a glicosilação, dando origem a um composto capaz de ser marcado radioactivamente para estudos biológicos.

Após ter sido concluída, com sucesso, a síntese dos análogos desejados, a avaliação da sua atividade antimicrobiana foi realizada por duas metodologias, diluição em ágar e microdiluição, tendo esta última sido escolhida para o apuramento de bioatividade de outros compostos.

Tendo sido obtidos os valores de CIM para todos os compostos sintetizados pelo grupo de pesquisa, foram estabelecidas as características estruturais essenciais para esta família de compostos. Com efeito, as estruturas e as características associadas à maior atividade antimicrobiana foram: (i) dodecil aglicona, (ii) dois grupos hidroxilo livres (iii) anómero α e (iv) *O*-glicósidos ou *C*-glicósidos. Todos estes atributos estão presentes no composto dodecil 2,6-didesoxi-α-L-arabino-hexopiranósido (**1**), que exibiu um valor de

CIM de 16 µg / mL, sendo este o composto ideal para prosseguir com os estudos biológicos.

Adicionalmente à determinação da atividade antimicrobiana do composto **1**, através de estudos focados nas suas potenciais aplicações, foi possível revelar que este composto não é eficaz na desinfeção de superfícies e não possui atividade esporicida. No entanto, mutantes bacterianos resistentes às principais famílias de antibióticos revelaram suscetibilidade inalterada a este composto (CIM = 16 µg / mL) e, para além disso, não se verificou nenhum desenvolvimento de resistência após a exposição contínua ao dodecil 2,6-didesoxi-α-L-*arabino*-hexopiranósido.

O estudo do mecanismo de ação foi iniciado empregando uma metodologia de time-kill de forma a avaliar os parâmetros cinéticos da ação bactericida observada. Em seguida o impacto do composto **1** na vitalidade bacteriana foi avaliado por citometria de fluxo, e foi observada uma população celular refratária, potencialmente resistente a estes compostos. Isto motivou a utilização de microscopia de fluorescência, recorrendo a uma estirpe bacteriana expressora de GFP, de forma a avaliar o impacto deste composto na vitalidade bacteriana ao longo do tempo. Uma vez completamente caracterizada a ação bactericida, a procura por potenciais alvos moleculares foi iniciada adaptando a tecnologia "Phenotype Biolog® microarray" para medir a resposta metabólica diferencial à exposição ao composto **1**, fornecendo informações sobre quais vias metabólicas potencialmente mais afetadas. Desta forma, o impacto em várias vias metabólicas foi determinado e estabelecendo-se um modelo de relação hierárquica entre elas, os alvos moleculares mais prováveis foram identificados como sendo os sistemas celulares de transporte ABC e PTS, e a biossíntese de aminoacil-t-RNA.

Assim, estudos genéticos foram iniciados, de forma a confirmar a validar os potenciais alvos detetados, com a criação de bibliotecas de mutantes por transposição aleatória, bem como uma biblioteca obtida por knockout específico dos sistemas de transporte, no entanto, nenhuma biblioteca testada mostrou alteração de suscetibilidade, o que indica que a existência de um alvo molecular específico é muito improvável.

Porém, uma vez que todos os hipotéticos alvos estão associados à membrana bacteriana, procurou-se avaliar a importância destas ultraestruturas para atividade antibacteriana. Foram criados protoplastos e esferoplastos bacterianos pela aplicação de lisozima e a determinação da sua susceptibilidade revelou que, no caso das bactérias

Gram-negativas, as ultraestruturas que envolvem a membrana citoplasmática são as responsáveis pela resistência à ação deste composto. Pelo contrário, nas bactérias Gram-positivas, elas não têm efeito, significando que a especificidade observada para *Bacillus* spp., exemplificada pela resistência de *S. aureus*, resulta das características da membrana bacteriana. A observação da ação do composto **1** no envelope celular foi efetuada utilizando técnicas de AFM, que demonstraram inequivocamente que estes compostos causam alterações na morfologia da superfície celular e danos celulares, mesmo em concentrações sub-inibitórias.

Em resumo, o trabalho desenvolvido permitiu estabelecer uma correlação entre a estrutura dos alquil desoxiglicósidos e a sua bioatividade. Complementarmente, foi possível demonstrar e caracterizar a ação bactericida que exibem, bem como, obter informações essenciais sobre as peculiaridades do seu mecanismo de ação, em particular, a aparente seletividade para o género *Bacillus*.

Assim, acreditamos que o trabalho desenvolvido e os resultados apresentados poderão representar um importante contributo para o desenvolvimento científico nas matérias que foram objeto de estudo, tanto no que respeita ao combate à progressiva resistência bacteriana, como em resposta à necessidade de novas ferramentas contra o Alzheimer.

Palavras-chave: multidisciplinar, glicósidos surfactantes, glicosilação, antibacteriana, modo de ação

Agradecimentos

Gostaria de começar por agradecer aos meus orientadores, que me orientaram não só no trabalho académico como a nível pessoal, contribuindo para a pessoa que sou hoje. Professora Amélia Pilar Rauter, cujo entusiasmo e força de vontade foram muitas vezes a força motriz do trabalho por mim desenvolvido, e por encorajar o meu espírito crítico e o desenvolvimento das minhas ideias, facultando-me preciosas oportunidades, sendo essencial para o cientista que sou hoje. Doutor Ricardo Dias, por me ter apoiado na minha imersão por terras desconhecidas e ter despertado em mim uma curiosidade científica sem limites, obrigando-me a enfrentar as barreiras do meu conhecimento, a pensar de forma diferente e a ver os problemas como oportunidades, e por ter sido, muitas vezes, mais do que meu orientador.

Ao longo deste trabalho tive a oportunidade de trabalhar com muitas outras pessoas que deixaram a sua marca, e às quais é igualmente devido um profundo agradecimento. Prof. Ana Viana por me orientar na pequena digressão pela microscopia de força atómica e por me dar apoio em várias etapas ao longo deste caminho. Prof. Nicola Colabufo, por me ter dado a oportunidade de uma experiência única em Itália, permitindo-me conhecer pessoas tão singulares como Tiziana, Mariangela, Ignazio, Simon, Alessandra, Mauro e tantos outros, assim como Vicenzo, com quem partilhei a minha estadia em Bari e que me mudou como pessoa. Dra. Alice Vieira, pelas infindáveis conversas sobre tudo e mais alguma coisa, e por ter sido, ao longo de tantos anos, e sobretudo, uma amiga com a qual pude sempre contar.

É com os colegas de trabalho que muitas vezes contamos para nos ajudarem a encontrar soluções e olharem para os nossos problemas de outra forma, ou muitas vezes simplesmente para partilharmos as dificuldades que surgem. A todos eles fica aqui um obrigado por todo o apoio, mas especialmente ao Rafael, Catarina e Luís, com os quais partilhei todo este trabalho e para o qual cada uma das suas participações foi essencial, assim como toda a partilha de ideias e apoio com todos os obstáculos que surgiram.

Andreia, nada disto teria sido possível sem ti, pois foste sempre o meu pilar e a minha fonte de motivação, pela pessoa forte, lutadora e visionária que és e com quem tenho a sorte de poder ter na minha vida. Por tudo isto não te poderia estar mais agradecido

por aceitares a minha companhia e me ajudares a ser uma pessoa melhor. Espero que possamos ter a companhia um do outro por longos anos vindouros.

Não há também agradecimentos suficientes para os meus pais, que sempre me apoiaram incondicionalmente em todas as minhas escolhas e sempre lutaram por me dar o melhor que podiam, muitas vezes em sacrifício próprio. É sem dúvida a eles que devo a pessoa que sou e a onde consegui chegar, pois sempre me motivaram a seguir os meus sonhos e a lutar pelo que quero. Por tudo isto e muito mais, é para eles esta vitória. Não posso deixar de agradecer também a todos os restantes membros da minha família pelo constante apoio e preocupação, apesar da distância.

A todos os meus amigos um muito especial obrigado, pelos incontáveis momentos de diversão, conversa e companhia que tornaram a vida em Lisboa muito mais agradável. Quero aqui deixar um especial agradecimento ao Nuno, Duarte e Inês, com os quais partilhei tantos momentos inesquecíveis e tantas voltas a vida já deu desde que vos conheci; ao Gabriel, Francisco e Mónica, por tantas conversas e companheirismo e que os jantares/almoços continuem por muitos, muitos anos; à Catarina e Tiago, sempre a postos, tanto para a diversão como para desabafar sobre uma pizza; e a tantos, tantos outros que fizeram e fazem parte da minha vida, muito obrigado, pois cada um de vocês participou neste processo ao me ajudarem e me apoiarem.

A todas as pessoas com quem cruzei caminhos, muito obrigado.

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List of Abbreviations

ABC - ATP-binding cassette

AD - Alzheimer's disease

AFM - Atomic force microscopy

ATCC - American type culture collection

ATP - Adenosine triphosphate

Aβ - Amyloid β

BBB - Blood-brain barrier

Cas - CRISPR-associated

CCCP - Carbonyl cyanide *m*-chlorophenyl hydrazone

CCY - Casein–Casein–Yeast

CFU - Colony forming units

CIM – Concentração inibitória mínima

CL - Cardiolipin

CM - Cytoplasmic membrane

CMC - Critical micelle concentration

CNS - Central nervous system

CRISPR - Clustered regularly interspaced short palindromic repeats

CV - Covariance

DCM - Dichloromethane

DIC - Differential interference contrast

DMAP - 4-Dimethylaminopyridine

DMEM - Dulbecco's modified eagle's medium

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

FSC - Forward scatter channel

FTICR - Fourier-transform ion cyclotron resonance

GFP - Green fluorescent protein

GLUT - Glucose transporter

GM – Geometric mean

IC_{50} - Half maximal inhibitory concentration

IM – Inner membrane

LC – Liquid chromatography

LDF - Low-density fibreboard

LPS - Lipopolysaccharide

LTA - Lipoteichoic acid

MBC - Minimal bactericidal concentration

MIC - Minimal inhibitory concentration

MS – Mass spectrometry

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAD - Nicotinamide adenine dinucleotide

NADPH - Nicotinamide adenine dinucleotide phosphate

Nd – Not determined

NIS – *N*-iodosuccinimide

NMDA - *N*-methyl-D-aspartic acid

NMR - Nuclear magnetic resonance

OD - Optical density

OECD - Organisation for economic co-operation and development

OM – Outer membrane

PAINS - Pan-assay interference compounds

PBS - Phosphate-buffered saline

PE - Phosphatidylethanolamine

PG - Phosphatidylglycerol

PMF - Proton motive force

PTS - Phosphotransferase system

P-gp - P-glycoprotein

RNA - Ribonucleic acid

TLC - Thin-layer chromatography

TPHB - Triphenylphosphane hydrobromide

UHPLC - Ultra-high performance liquid chromatography

UV-Vis - Ultraviolet-visible spectroscopy

Chapter I – Introduction

1 Antimicrobial resistance and the path forward

1.1 Antibiotics and the emergence of resistance

The discovery of antibiotics was a stepping stone in human evolution, since until the middle of the nineteenth century, infectious diseases were the main cause of death in the developed world[1]. The accidental discovery of penicillin in 1929[2] and the later development of sulfonamides[3] changed that, and since then, modern medicine has been dependent on antibiotics and vaccination as the main weapons in fighting off infection.

Although the first antibiotic largely used was of synthetic origin, a sulfonamide named prontosil discovered in 1932[3,4], the clear majority of antibiotics discovered were obtained from Nature or derived from them. The discovery of natural antibacterial compounds began fortuitously with penicillin, but the development of a discovery platform by Selman Waksman[4], revolutionized the search for antibiotics. This "Waksman platform" was widely adopted by the pharmaceutical industry, leading to the golden era of antibiotic discovery, where most of the major classes of these drugs were obtained from microorganisms, namely, aminoglycosides (streptomycin, from *Streptomyces griseus* in 1943), amphenicols (chloramphenicol, from *Streptomyces venezuelae* in 1947), cephalosporins (cephalosporin, from *Acremonium sp.* in 1945), glycopeptides (vancomycin, from *Amycolatopsis orientalis* in 1953), macrolides (erythromycin, form *Saccharopolyspora erythraea* in 1952), penicillins (penicillin, from *Penicillium chrysogenum* in 1928) and tetracyclines (chlortetracycline, from *Streptomyces aureofaciens* in 1945)[3–6].

From the 40s to the 60s, a great number of antibiotics were revealed and immensely used, but natural product discovery quickly started declining, until it became impractical due to the increasing difficulty of identifying new classes of antibiotics against the background of known compounds[7].

At the same time antibiotic discoveries were made, bacterial resistance was also documented. Resistance to penicillin was detected right after its introduction in 1947, due to the emergence of β-lactamase causing the hydrolysis of the drug[8]. This could be recalled as the first call of attention to an emerging problem, that was observed in all the early used antibacterial compounds[4] and culminating in one of the major modern health threats. Since natural selection has provided a stream of bioactive small molecules with

antibiotic properties it can be expected that the same natural selection, or evolutive pressure, would provide the tools to overcome said molecules. This happens to be the truth, as it was later discovered by genetical analysis. Microorganisms always had the molecular tools to drive resistance, as shown by surveys from all kinds of environmental niches and even from samples found in permafrost[9,10]. This collection of antimicrobial resistance genes was designated resistome and it pre-dates the modern antibiotic era by far[11].

The resistome consists of a collection of resistance genes in a given environment and encompasses both intrinsic and acquired resistance genes, proto-resistance genes and cryptic resistance genes[12].

Intrinsic genes refer to the inherent antibacterial resistance and that is independent from previous exposure to antimicrobial compounds. These are present in many bacterial species, pathogenic and non-pathogenic, and are most commonly associated to reduced permeability of the bacterial envelope and the presence of multidrug efflux pumps. Generally, these genes are also associated to other essential biological roles for the cellular function[13,14].

Acquired resistance genes refers to the bacterial resistance observed in species that were susceptible to the antibacterial drugs, but by being in a dense bacterial population with resistant bacteria, they acquire the genetic material necessary to express reduced susceptibility. This acquisition is performed by horizontal gene transfer, in which by a number of diverse mobile genetic elements (like plasmids, transposons and integrons), the genetic material is passed between bacteria, mostly of the same species, but also from another genus and even other families. The most common resistance mechanisms that can be acquired are the target modification and antibiotic inactivation[15].

Lastly, proto-resistance[16] and cryptic[12,17] resistance genes are those that do not confer antibacterial resistance, but these are genes that through mutation or selective pressure can be activated to give rise to an antimicrobial resistant phenotype. Cryptic genes are often not expressed or with a very low expression, lacking an obvious association to antibiotic resistance, while proto-resistance genes are those that have the potential to evolve into resistance genes.

Our increasing knowledge of the resistome is also modifying the way we think about the emergence of antibiotic resistance and the therapeutic approaches to be developed in order to overcome this issue[12].

Meanwhile, antibiotics are hardly exclusively for human use, and it is their application in a massive scale in livestock and poultry production that allows this enormous number of animals to resist disease in such dense populations[18,19]. In fact, in many countries, antimicrobial compounds are used in larger volumes in the animal production industry than for human consumption, and, for this reason, it is considered an important factor driving the current antimicrobial resistance crisis[18,20,21]. This incessant use in these industries, as well as in human health care, has created extraordinary conditions for the deployment of genetic resistance tools, over time causing the selection of evermore drug-resistant bacteria.

The emergence of resistance is also driven by an vital forgotten fact about antibiotic therapy: the drugs employed are mainly effective in rapidly growing bacteria. However, during the course of treatment, a fractional population survives to treatment, designated as persister cells[22,23].

Persisters are not mutants but phenotypic variants produced stochastically in the population, known to be formed by all known pathogens.[24] These dormant or non-replicating bacteria are tolerant to antibiotics, but once the concentration of antibiotic falls, they resume growth. The number of persisters in each population varies greatly, depending in the age of the bacterial culture, the type of antibiotic, the concentration and length of exposure, medium composition and other environmental factors.

During the course of treatment, antibiotics are used and effectively kill most of the bacterial population causing the infection, but the remainder will possibly consist in a high number of persister cells. Additionally, the macrophage action that ensues any infection was observed to greatly contribute to the formation of persister cells[25], and persisters that give rise to highly antibiotic resistant descendants. The fact that macrophage phagocytosis places the bacteria in a nutrient-deprived, aggressive environment seems to potentiate the formation of persister cells and activation of toxicity-resistance mechanisms.

Even though the environmental clues and cellular mechanisms that lead to the formation of persisters are poorly understood, the connection between the persister population and the emergence of antibiotic-resistant bacteria cannot be denied.[23,24]

1.2 Past and Future of antibiotic discovery

Antibiotic discovery started with the synthesis of Prontosil, a red dye product of a trial-and-error synthetic effort by Bayer[26]. This drug revolutionized healthcare when introduced in 1936 and is responsible for many saved lives during the Second World War. This spurred antibiotic research leading manufacturers to produce countless variations of the drug, some with dire consequences due the lack of testing requirements.

Penicillin, that was discovered in 1928, was not used until 1939 and even then, treatment was still in a research setting, since its isolation in large quantities was difficult and supplies were scarce. Challenges to its mass production and subsequent application continued until mid-40s[27].

Nonetheless, the pharmaceutical industry was increasingly interested in discovering new antimicrobial compounds and several compounds of natural origin emerged as a response. The development of tools like the "Waksman platform", allowed a systematic study of samples, a very different approach from the fortuitous discovery as in the case of penicillin. This platform consisted in the screening of soil samples for microorganisms that showed antimicrobial activity against a test organism by detecting areas of growth inhibition on an overlay plate[28]. Its introduction and vast application by the pharmaceutical industry lead us to the golden age of antibiotic discovery, as referred before.

As soon as new antibiotics were discovered, a new problem was raised with resistance starting to be observed for all of them (Figure 1), thereby motivating the development of synthetically modified derivatives of the original scaffolds. The most successful cases of drug development were indeed obtained by creating synthetic analogues to natural scaffolds from the golden era of discovery, but even if these derivatives proved to be very successful when they were developed, by the end of the 20th century, the escalating bacterial resistance was a clear indicator that the race was lost[29].

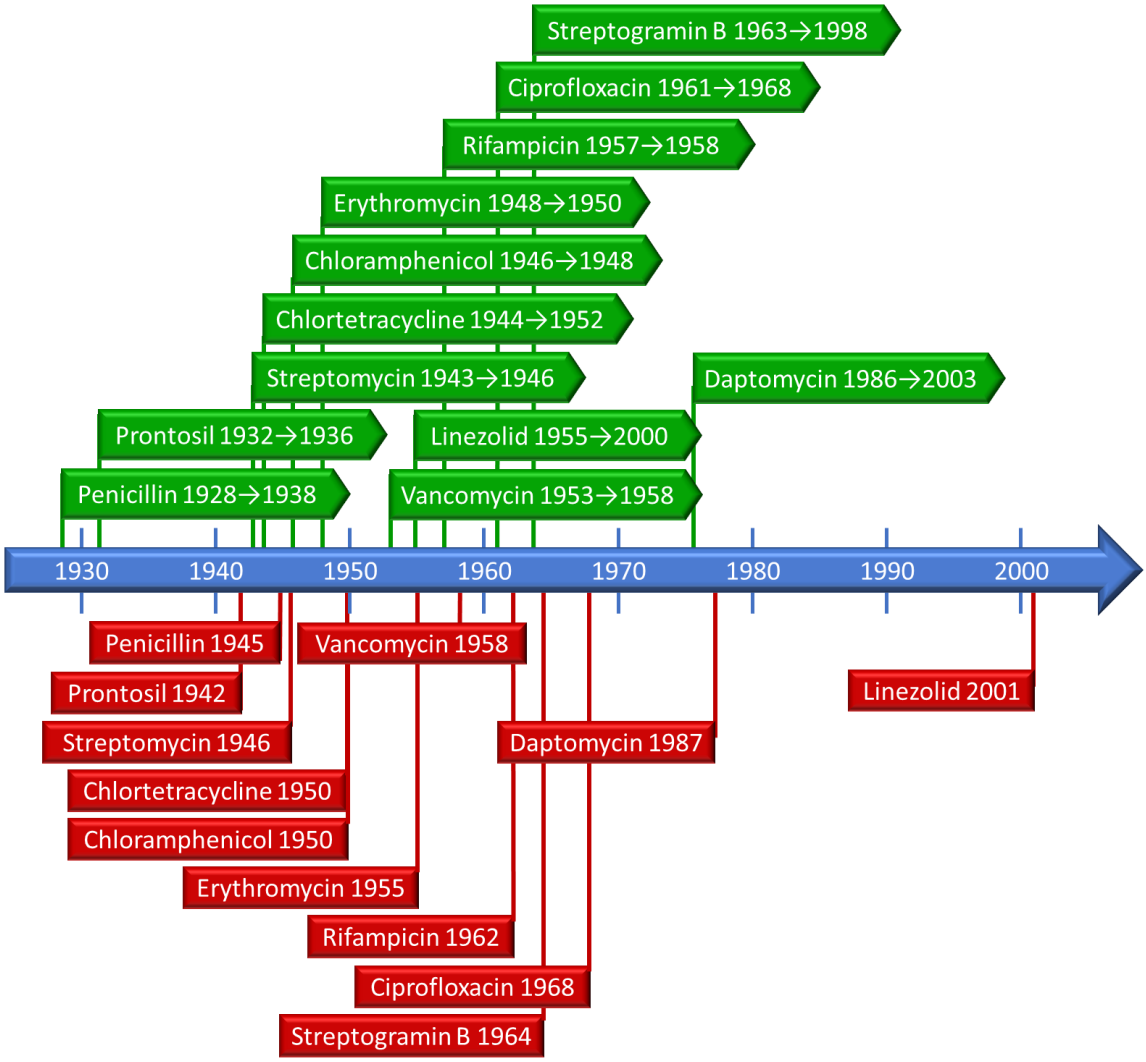


Figure 1. Representative time-line of the year of discovery and application (in green) and detection of resistance (in red) for the first discovered antibiotic of each main class.

Additionally, the number of large pharmaceutical companies actively researching novel antimicrobial compounds is in decline since the 80's[30,31], possibly a major factor in the lack of appropriate response to such developing issue. The development of therapies for other clinical indications that demand longer therapy times and the increased profitability that is associated are a major driving force of this trend.

After the gold age of antibiotic discovery, a stale period ensued, despite several different approaches being undertaken, but the truth is that from the 70's to the 90's very few successful new antibiotics have been introduced in the market[5].

The methods utilized for natural product screening have evolved, but the most commonly produced antibacterial compounds have already been identified. Nonetheless, a continuous effort exists in searching for potential new sources of antimicrobial natural compounds, such as from marine invertebrates and uncultured bacteria[5,32], as well as by

the modification of previously known compounds to analogues capable of overcoming the antibacterial resistance. This rational synthetic effort is supported by the increasing knowledge of the antimicrobial mechanism of action and respective mechanisms of resistance. In fact, natural products or their derivatives still constitute a substantial part of compounds in clinical trials[33].

However, this approach has another profound issue associated: while Nature can provide an enormous variety of molecules, the fact is that these compounds may not provide an answer to the problem we are facing, if they do not act under novel mechanisms of action. This is of critical importance due to the well-known phenomena of cross resistance between compounds of the same class of antibiotics[5,34].

The development of genetics and biotechnology allowed the establishment of new platforms for drug research[29,35]. Likewise, developments in high-throughput synthesis allowed the creation of large chemical libraries, which could be subjected to computational methods of analysis and high-throughput biochemical screening assays. But most of the research has been predominantly guided by metrics such as the Lipinski rule of five, for orally available drugs[36], optimizing them to the human biology, even though most antibiotics in clinical use do not conform to those parameters[37]. At the same time, genomics searched for conserved essential proteins, as the major targets to focus high-throughput synthesis and rational design, but even if and when inhibitors were identified, the access to those targets was not achieved or ineffective. In fact, these approaches produced very poor results.[38]

Nowadays, some new tools are emerging in the fight against bacterial resistance, but instead of being guided by technological advancement, but rather by a new way of looking to old approaches. The idea of a single small-molecule that interacts with a single target has been mostly abandoned, since the most successful drugs are actually multi-targeted, and combinatorial and adjuvant approaches offer many advantages over the traditional methodologies[39]. Likewise, instead of a broad-spectrum approach, a species-selective could have a very favourable outcome in many diseases, being capable of preserving microbiota, as well as diminishing some of the selective pressure in non-pathogenic bacteria.[5,40]

1.2.1 New antibiotic discovery methodologies

Although today there is a renewed interest in antibiotic discovery and increased activity in this field, the past 30 years were of low outputs[41]. One of the already referred reasons for this was the diminishing interest in the search for natural compounds, in favour of synthetic discovery, with the screening of large compound libraries. But the ineffectiveness of this approach is pushing the research back to natural products, and new techniques were developed to allow the study of previously unculturable microorganisms[42,43].

Likewise, advances in biotechnology, namely in genomics and metabolomics, gave rise to new tools to obtain information about possible biological relevant secondary metabolites, without the need for the microorganism growth, compound production and isolation, relying instead mainly in *in silico* approaches[34,44].

1.2.1.1 Methodologies for the discovery of new natural compounds

These new sources of natural compounds became available due to the development of several new discovery platforms and methodologies, a revival of the "Waksman platforms". One of these techniques is in situ cultivation, which involves the growth of microorganisms in the presence of a simulated natural environment. In one of the earlier accounts of this method, Kaeberlain et al. (2002)[45] used diffusion chambers to mimic the marine environment and were able to culture two new bacteria. This technology developed into the iChip (isolation chip), a high-throughput screening system of miniaturized diffuse chambers, that by being placed in the natural environment allow for naturally occurring compounds to freely diffuse in the growth media, serving as nutrients for the target microorganism, imitating its natural environment in a single colony format. Such methodologies already enabled the discovery of new antibiotics, namely, lassomycin and teixobactin[43,46,47].

Another emerging approach is co-culture of microorganisms, where the co-cultivation of two or more microorganisms will lead to an influence in growth and the activation of new biosynthetic pathways, from the presence of signaling bioactive molecules in the media. Using this method, a new alkaloid, aspergicin, was isolated by Zhu et al. (2011)[48]. (a current overview of this technique was performed by Beppu in 2017[49], detailing the specific variables and promotive factors for the co-cultivation of several microbial strains.)

These approaches must also be paired with suitable identification technologies, that are also showing important evolution. Advances in analytical methods such as nuclear magnetic resonance (NMR) and mass spectrometry (MS) allow for database-associated comparison between possible new compounds and the ones already discovered, providing at least a reasonable guess to the novelty of a compound.

This rediscovery problem can also be avoided by taking in silico approaches in the selection of microorganisms or compound discovery. One main approach receiving important attention is genome mining[34]. This poses an alternative to the microorganism production and posterior analysis of secondary metabolites by directly looking at the sequenced genome and determining if any biosynthetic gene clusters involved in the production of new antibiotics can be found. A number of bioinformatic tools were already used in the discovery of new secondary metabolites: DECIPHER, antiSMASH or BAGEL are just examples[50–52]. Nonetheless, an important limitation to the methodology is that it is based in the comparison to known biosynthetic pathways, meaning it will fail to detect completely new ones, and is also limited to the effectiveness of its prediction capabilities. Still, novel approaches emerge to overcome the limitations and the development of metagenomics as of other culture-independent methods show promise in their capabilities[53].

Metabolomics, with the development of associated analytical methodologies such as UHPLC, FTICR or LC-NMR-MS, is also of growing importance, being ideal for the screening and subsequent comparison of secondary metabolites produced by microorganisms[43,54]. Such techniques provide structural information of compounds in trace amounts making them useful in identifying potentially valuable biomolecules. This capability associated with the database structural comparison allows for rapid discrimination against previously known metabolites, preventing redundant analyses.

1.2.1.2 Sources for new natural compounds

The increased interest in the discovery of new antimicrobial compounds resulted from the recognition of the existence of numerous untapped potential sources of bioactive molecules.

The marine environment is full of microorganisms and its exploration is a very recent development, but already provided results as already described before. Marine actinomycete species are considered an important source of novel bioactive secondary

metabolites. Marine sponges and corals are also on the spotlight, providing a plethora of secondary metabolites, many of them originated from bacteria colonizing these animals[55,56].

Another realm of microorganism with rising popularity are endophytes, microorganisms, mainly bacteria and fungi, living symbiotically with plant species for at least some of their life cycles. The relationship between these microorganisms is not yet well understood, but recent reports[57,58] show endophytes as a rich reservoir of natural products with antimicrobial activity. Since this is a vastly underexplored realm of microorganism, the probabilities of uncovering novel antimicrobials are promising.

Other sources such as fungi or plants, traditional sources of important bioactive compounds, are also relevant and their study continues, but many newly discovered compounds are toxic also to eukaryotic cells, derived from the broad antimicrobial activity these organisms use as a defense mechanism[5].

1.2.1.3 Other approaches to antibiotic development

Synthetic methodologies are also observing important advances, since new natural compounds represent new scaffolds capable of synthetic improvement. In fact, such remarkable improvements in the discovery of novel natural compounds and their biosynthesis lead to the development of a new synthetic approach: combinatorial biosynthesis.

Using this approach, instead of a very laborious and costly development of a complete synthesis, either by chemical or chemoenzymatic routes, genetic tools are used to modify the genes involved in the biosynthesis of the natural products, leading to the production of non-natural compounds *in vivo*. This technique has already seen some cases of success, for example with telavancin[59], and its application to sugar-containing antibiotics is a recent focus[60].

1.2.1.3.1 Combinatorial and adjuvant approaches

An increasing understanding that the most successful antibiotics interact with multiple biological targets has motivated the study of possible combinatorial approaches. The combination of antibiotics that is used nowadays was mainly a product of clinical experimentation and focused in acting against a broader spectrum of pathogens, when facing an unknown infectious agent, a synergetic activity or to suppress the emergence of resistance. What has gained increased interest is the study of possible combinations that,

although not necessarily synergistic, will act in multiple cellular targets, increasing the effectiveness of therapy and reducing the resistance emergence.[41,61]

Adjuvants are another type of combinations, where a non-antibiotic compound inhibits some enzymatic target or biological process, effectively potentiating the activity of an antibiotic with which it is combined. A good example of this is the combination of amoxicillin with clavulanic acid, which inhibits the b-lactamase drug resistance enzymes. With our increasing knowledge of the antibiotic resistance mechanisms, such approaches will be of increasing value, by developing molecules capable of returning antimicrobial activity to known drugs[39,62].

1.2.1.4 Species-selective antibiotics

A narrow spectrum of activity has been traditionally considered a big disadvantage in antibiotic discovery, and narrowly selective compounds identified using high-throughput screening platforms, were mostly discarded do to this same characteristic, in favor of wider-spectrum alternatives. Nonetheless, the study of the mechanism of action of some species-selective compounds revealed that they tend to focus metabolic targets well-conserved within the species or group[63,64].

However, species-selective compounds are now considered to be the future of antibiotic discovery[5,65], due to the many, now considered, advantages this approach contains. Firstly, the search of selective compounds eliminates nuisance compounds as the pan-assay interference compounds (PAINS), which by their surfactant or DNA intercalating properties have a broad spectrum of activities[66]. At the same time, such compounds of narrow-spectrum of activity are also not likely to be toxic to humans, since the hypothetical targets present in a strict group of microorganisms are very unlikely to be present in humans.

Resistance development is also hindered by a species-specific approach, because when using broad spectrum antibiotics, if one species develops a resistant mechanism, it can pass it onto others by lateral gene transfer. Such processes would be inviable with such antibiotics.[40,67]

Another very important advantage of a species-selective therapy is the preservation of our microbiota during the course of treatment. Our gut microbiome is now recognized as a major player in human health, both physically and mentally, and so its preservation is paramount to future therapies. Additionally, specific antibiotics would

allow not only to protect the microbiome but also to manipulate it. This is of major interest, since nowadays the best methodology to positively impact an unhealthy microbiome is by fecal microbiota transplantation. With the development of selective antimicrobials, the suppression of unfavorable species could be achieved, leading to a cure for a number of gastrointestinal diseases and even neurological conditions[68,69].

1.2.2 Alternatives to antibiotics

With the difficulties presented by the development of new antibiotics, other approaches have emerged with growing interest. One of such approaches is the development of antivirulence methodologies either using antibodies or small-molecule inhibitors of virulence mechanisms. Since this approach poses little or no selective pressure in the microorganisms, the emergence of resistance should be much lower than with traditional therapy.[70,71]

Another emerging possibility is the utilization of RNA-guided nucleases of CAS/CRISPR (clustered regularly-interspaced short palindromic repeats). Such systems that recognize unique DNA sequences could target and kill specific pathogens or inactive resistance mechanisms. The main limitation for this approach resides in a suitable delivery method, generally involving bacteriophage vectors.[72]

1.3 Biological Targets

Traditional antimicrobial compounds resulted from screening efforts, and so, their target was studied after proven efficacy. But in the past decade, with the development of genetics, the possibility of finding possible targets first, and then develop molecules capable of interacting with them became the main paradigm in antibiotic research. Genomics and the essential gene search were acclaimed as the tools that would provide us with an abundance of new antibiotics, but, as was already discussed, such approach was a failure. Certainly, multiple factors are responsible for this, and overcoming this issue will most likely require a paradigm shift in the way we use modern tools and a redefinition of what we consider druggable targets.

The fact remains that current antibiotic therapy targets only a small subset of microbial essential processes, and most new antibiotics developed are focused in

incremental modifications of known drugs, and so, focusing on the same targets.[73] In recent years, only a single new target has emerged with impact in antibiotic research: a cell-wall synthesis component, lipid II[74].

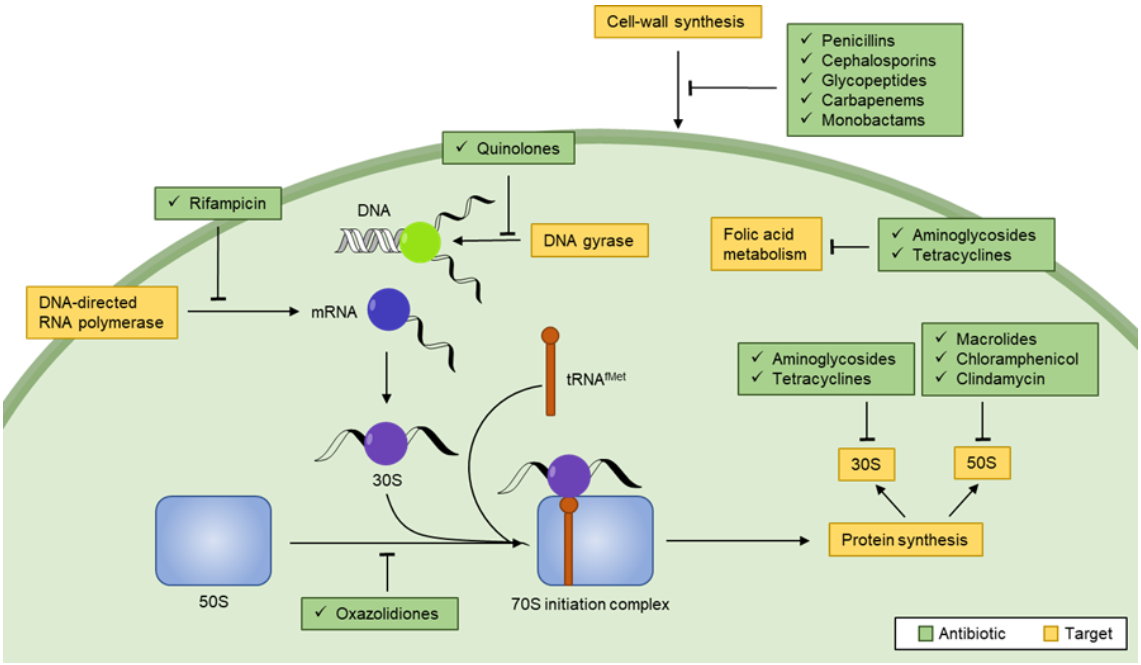


Figure 2. The major targets of antibiotic action

Nonetheless, the search in the genome for essential genes has provided numerous new antibacterial targets of potential utility[33], however it is evident that the idea that any gene required for microbial growth creates a plausible drug target is incorrect. The successful prioritization of the new "possible targets" is one of the major necessities for the successful implementation of these tools. At the same time, the conditional context of gene essentiality is being overlooked, possibly neglecting valuable targets as well[38,73].

Since the search for new antimicrobials is focused in *in vitro* activity, without consideration for the host-pathogen context, this also represents an opportunity for possible new targets related to host-pathogen interaction and bacterial infection. Additionally, broad-spectrum activity is also preferred and screened for, resulting in the discard of new narrow spectrum antimicrobials with possibly new mechanisms of action, due to highly specific targets.

With the current abundance of possible targets and no well-defined method of prioritization, several studies and literature reviews are emerging aiming to sort out this problem[73,75–77].

One transversal issue to most of the "new targets" resides in the fact that they are present in the cytoplasm and many small-molecule inhibitor hits fail to enter the bacterial cell, possibly because the "Lipinski rules" that heavily influenced the physicochemical profiles of most industrial chemical libraries are not optimized for antibiotics. This was noted by Lipinski himself[78], since antibiotics were outliers from the orally active compounds used to formulate the rule. Given this, a compilation of rules or exceptions could be derived from studying known antibiotics, and it would be a better guide to bacterial permeation.

1.3.1 The bacterial cell envelope

The bacterial cell envelope is the gateway to the cell for all exogenous compounds and, as such, is one of the main factors impacting the success of antibiotic therapy. These structures are fundamentally different from thouse of animal eukaryotic cell cells (that consists of only a plasma membrane), providing good inherent specificity to drugs that target them.

Bacteria are generally classified in two main groups, the Gram positive and Gram negative, depending on their Gram-stain retention properties, a classification based in the marked differences in their ultrastructures.

Gram positive bacteria have a simpler bacterial envelope, consisting of a cytoplasmic membrane (CM) surrounded by the cell wall. This outer layer is comprised mainly of peptidoglycan (a polymer constituted by a carbohydrate backbone and amino acid residues) forming a thick mesh, anchored to the membrane by lipoteichoic acid (LTA) strands.[79,80] Between these two ultrastructures exists a small periplasmic space (Figure 3).

The more complex Gram-negative bacteria possess two membranes, an outer membrane (OM) and an inner membrane (IM), and the periplasmic space in between them, where a small cell wall is present, also constituted by peptidoglycan (Figure 3). The OM is an asymmetric lipid bilayer, with the outer leaflet being constituted mainly by lipopolysaccharides (LPS)[81], and the inner leaflet by glycerophospholipids. The LPS content is responsible for the low permeability of the membrane and is one of the main obstacles to the passage of drugs. Passage through this membrane is mostly mediated by the presence of porins.[80,82]

The IM is constituted mainly by phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL), much like the CM, apart from the presence of LTA in the latter. In these membranes, the lipids' proportion vary greatly according to the bacterial species (as well as the lipid composition, with some species having unique lipids), but generally Gram-negative bacteria are richer in PE[83].

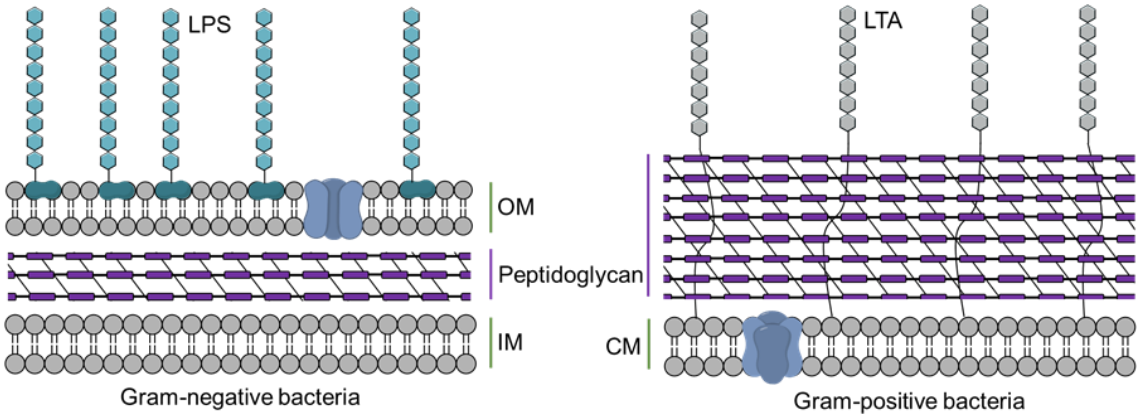


Figure 3. Schematic presentation of the membrane architecture of Gram-negative and Gram-positive bacteria.

1.3.1.1 Membranes as antimicrobial targets

Antimicrobial agents targeting the cell wall are widely used, as all β-lactam antibiotics act by inhibiting cell wall synthesis, but bacterial resistance to these antibiotics is widely spread and quickly acquired[4]. That is why pursuing other biological targets is essential. Additionally, for the activity of most drugs and their access to the bacterial cytoplasm, the peptidoglycan does not represent a barrier, and so, the membrane emerges as an attractive target.

Some drugs exploit the presence of anionic lipids on the surface of bacterial membranes, specially Gram-negatives, as means of displaying increased selectivity, for example, aminoglycosides, that display multiple cationic moieties in physiological conditions, and are even actively transported into Gram-negative bacteria[80]. Many membrane-active antibiotics display the same strategy[83,84], whether by specifically targeting certain lipids (like daptomycin, that exhibits an PG-dependent insertion on the membrane) or by using electrostatic interactions to guide their surfactant action to the bacterial membrane (e.g. polymyxins, that link to the LPS and then their aliphatic moiety destabilizes the bacterial membrane).

The factors that affect the action of membrane-active antibiotics are not limited to bacterial surface charge, covering also the more general amphiphilicity of the membrane, as well as other physical properties like fluidity/packing, curvature and clustering of lipids[83]. Since these are core properties of bacterial membranes, they are associated to the multiple phenomena that can lead to bacterial death when under the action of surfactant agents. The disruption of the cell membrane bulk biophysical properties, by altering the lipid packing, clustering or curvature, leads to membrane reorganization and/or permeability alteration, and consequently an alteration of the proton motive force (PMF), and even the destabilization and/or loss of function of membrane embedded proteins.

Given the properties discussed, membrane-active antimicrobials present several benefits, such as the fact that their target is essential and preserved among various bacterial species, with various degrees of similarity, possibly allowing for selectivity tuning. Also, they can be active against slow-growing or dormant bacteria (persisters for example) as well as in biofilms and display a low potential for the development of resistance, probably resulting from the multi-targeted mode of action. In general, they possess a favourable pharmacokinetic profile, and have displayed a great potential to serve as chemosensitizer, increasing the activity of other antibiotics[80,83].

However, the same properties represent some drawbacks of this type of approach, with the accessibility of the bacterial membranes and selectivity over animal cells representing some major concerns, and the reason why surfactant compounds are known and feared for their promiscuity that leads to undesired secondary effects.[80,83]

Nonetheless, the interest in developing compounds active by membrane-associated interactions or targets is growing and several new compounds are showing promising results[80,85]. Additionally, membrane-active antibiotic classes like lipopeptides, lipoglycopeptides and polymyxins (non-ribosomal peptides) are already in use, and the study of the mechanism of action of several members of this type of antibiotics revealed that many possess other targets in addition to the bacterial membrane (examples are daptomycin that also inhibits several biosynthetic pathways[86], or telavancin that also inhibits the transglycosylation of peptidoglycan cell wall synthesis[87]).

The introduction of amphiphilicity in already known antibiotics and the development of derivatives with a membrane-active moiety is an approach that can aid in overcoming antibiotic resistance, telavancin being one great example of success (resulting from the conjugation of vancomycin with a lipid moiety), but it is still an

underexplored approach. Lipid conjugation to aminoglycoside antibiotics is one of the emerging tendencies of this approach[80,88].

1.4 Carbohydrate contribution to antibiotic therapy

Most carbohydrate-based antimicrobial drugs in use nowadays are of natural or semisynthetic origin, and constitute some of the major classes of antibiotics, such as aminoglycosides or macrolides. Nonetheless, most highly successful antimicrobial drugs like erythromycin, vancomycin or gentamycin, even if they represent different antibiotic classes, they have the same general mechanism of action: antimicrobial effect due to inhibition of protein synthesis. This can be interpreted as a sign that these compounds contain a vast untapped potential, since the single mechanism of action does not translate the enormousness of diverse biological roles associated to carbohydrates.

The design of new carbohydrate-based antimicrobials has received special attention in drug discovery due to the increasing need of novel antibiotics, with novel mechanisms of action.[89,90] The exploration of other cellular structures as potential targets, such as the bacterial cell wall or membrane has gained increasing relevance for this reason.

Carbohydrates also display important therapeutic roles, not as a drug but as a potentiating system for available antibiotics. One example of this are host-guest complexation strategies, capable of enhancing the solubility, stability permeability and bioavailability of a guest drug[91,92]. These host-guest complexation methodologies rely in the non-covalent bonding between two molecules, and carbohydrates such as cyclodextrin are a prime example of the capabilities of this technique with applications in sensor, biomedical and pharmaceutical technologies[93,94]. Additionally, by applying these systems to antibiotic delivery it is possible to counteract the antibiotic resistance mechanisms, such as enzymatic degradation or efflux systems and even raise the affinity for modified targets by altering antibiotic conformation[95].

But carbohydrates and their ubiquitous in living organisms are also being studied as possible tools aiding the fight on antimicrobial resistance in multiple of ways: from wound dressing technology, since the risk of wound infection[96] is a major concern in multiple cases, to the use of cell-surface carbohydrate recognition as an infection diagnostic tool[97]. Also, therapeutic alternatives already referred such as vaccines and anti-adhesion therapies are highly reliant in carbohydrates.

1.4.1 Alkyl glycosides and their antimicrobial potential

Since the 70's the antimicrobial properties of saturated fatty acids and fatty alcohols are well known and were already in use[98,99]. Most of the early efforts to improve their application potential via conjugation were promoted by the oil industry, as a possible expansion on the use of oleochemicals as antimicrobial agents[100].

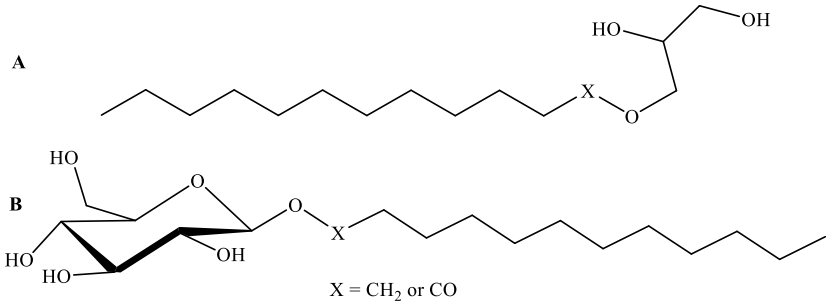


Figure 4. Exemplar structural representation of glycerol (A) and glucose (B) conjugates

The most abundantly studied conjugates were with glycerol and glucose (Figure 4), but also with other mono- and oligosaccharides. These conjugates were used as surfactants in cleaning solutions and cosmetics but also played roles as preservatives and emulsifiers[101–104]. Another important role of an alkyl glycoside (more specifically octyl glucoside) was in the solubilization of membrane proteins while retaining their activity[105,106].

The antimicrobial and surfactant capabilities of alkyl glycosides gained renewed notoriety in the 90's, with the increasing environmental concern, since such compounds are generally of low toxicity and biodegradable and both the sugar moieties and the alkyl chains could be obtained from renewable sources[107].

Several studies on their surfactant and antimicrobial properties show the great potential of these compounds and will be discussed in greater detail in chapter 3.1. Alkyl glycosides have been continuously used in cosmetics, as preservatives and in cleaning and disinfection solutions, but also in drug formulations showing the potential as druggable compounds[103,108,109].

2 Carbohydrate chemistry as an answer

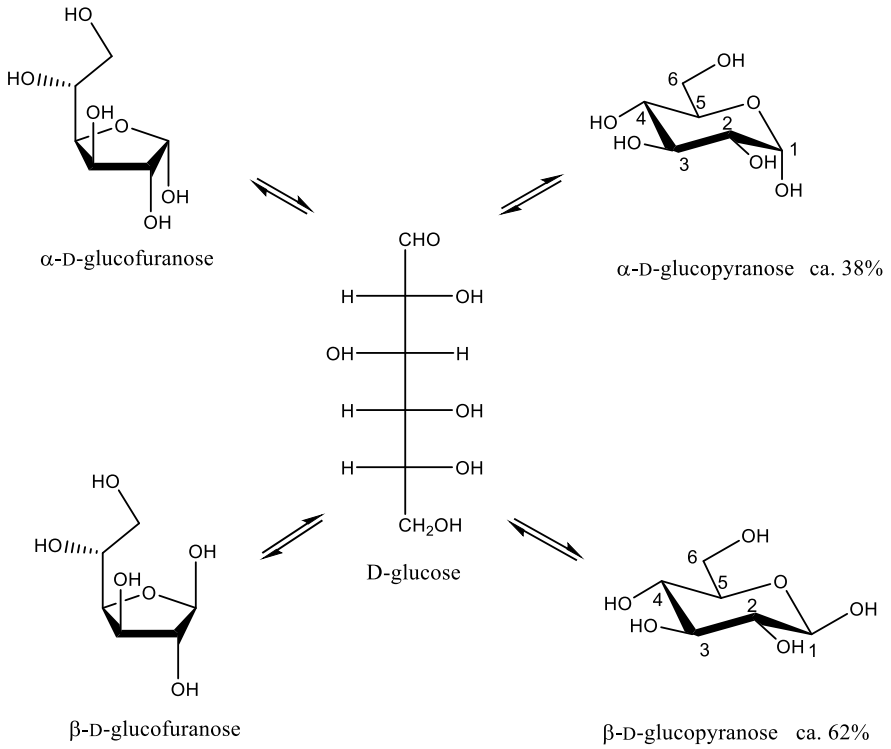
Carbohydrates are a class of compounds with the unique feature of exhibiting multifunctionalization, being one of the most abundant types of compounds in Nature. They are ubiquitous in the biological processes of all forms of life and their presence helped fuel traditional medicine with natural compounds, as well as providing valuable scaffolds for synthetic drug discovery. In fact, carbohydrates are regarded as important sustainable raw materials, and the increasing knowledge of their involvement on biological processes demonstrates the vast chemical space that is available for further exploration[89].

Carbohydrate chemistry is a promising area of research in the forthcoming years, as the role of carbohydrates has become more relevant specially in signaling and recognition, leading to important advances in immunotherapy and protein therapeutics.

2.1 Carbohydrate fundamentals

The term 'carbohydrate' (French 'hydrate de carbone') was applied originally to monosaccharides, in recognition of the fact that their empirical composition can be expressed as $C_n(H_2O)_n$. However, the term is now used generically in a wider sense. The generic term 'carbohydrate' includes monosaccharides, oligosaccharides and polysaccharides as well as substances derived from monosaccharides by reduction of the carbonyl group, by oxidation of one or more terminal groups, or by replacement of one or more hydroxy group(s) by a hydrogen atom, an amino group, a thiol group or similar heteroatomic groups. It also includes derivatives of these compounds. The term 'sugar' is frequently applied to monosaccharides and lower oligosaccharides[110].

Carbohydrates are subjected to a functional isomerization when in solution (Scheme 1). This isomerization from acyclic monosaccharide to the heterocyclic ring can be interrupted, if the anomeric position (the hemiacetal marked 1 in scheme 1) is conjugated to any other molecule, since the acetal functionality then formed is not converted into the corresponding ketone or aldose. In nature, carbohydrates are often found in this glycoside form, in which the sugar residues are stable maintaining its multifunctional potential[111].



Scheme 1. Acyclic and cyclic forms of D-glucose in water at pH 7

Carbohydrates are also stereochemically rich molecules and all ring carbon atoms of a cyclic saccharide are chirality centers, presenting one of the two configurations (*R*, *S*). Given this asymmetry, several isomers exist, e.g. 16 for a carbohydrate having 4 chirality centers, all of them being stereoisomers. Additionally, all have several conformers possible, for example, for pyranose the most common are the chair ($^1C_4$ and $^4C_1$) and boat conformations, but many more exist[112].

Monosaccharides are classified in two series, designated by the symbols D or L. Assignment of a monosaccharide to any of these series relies on the orientation of the projection of the bond linking the chirality center furthest from the carbonyl group to the hydroxy group, in the Fischer projection. If that hydroxy group is on the right, the molecule belongs to the D series, and accordingly if it is oriented to the left, the molecule belongs to the L series. The new chirality center, generated by the cyclization, also gives rise to two diastereoisomers, called anomers. In hexopyranosides, anomeric *α*- or *β*-configuration is defined by a *trans* or *cis* orientation of the anomeric OR group in relation to the hydroxymethyl group linked to C-5, respectively.

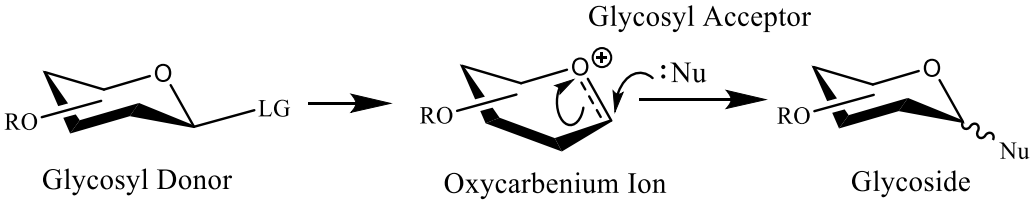
All these characteristics of carbohydrate core structures, with defined three-dimensional orientation of their substituents, provide them with unique capabilities in biological recognition systems[113,114].

2.2 Glycosylation

Sugar moieties in Nature are typically found as oligo- and polysaccharides or glycoconjugates comprising a variety of biological molecules. The most frequent linkage between monosaccharide residue and aglycone is designated glycosidic bond, an acetal functionality that can be obtained by a glycosylation reaction[115]. In Nature these glycosidic bonds are formed in highly regulated systems using enzymes, ensuring this way a high selectivity and efficiency. Chemists, on the other hand, must rely on extensive multi-step procedures in order to obtain glycosylated derivatives with the desired anomeric configuration, often at the cost of efficiency (if total yield is taken as an efficiency marker for starting material / product transformation). Such studies nonetheless gave rise to numerous procedures that can be used to achieve an impressive variety of products[116,117].

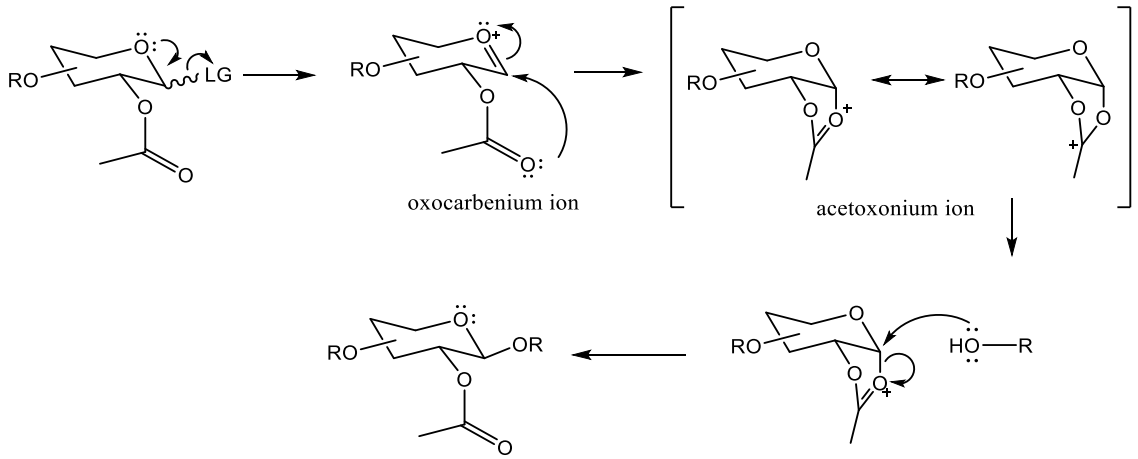
Despite the enormous variety of systems, all glycosylation reactions involve a glycosyl donor with a leaving group in its anomeric position that will react with a glycosyl acceptor in the presence of an activator, often with the formation of an oxacarbenium ion[118] (Scheme 2). One could be fooled by the simplicity of such description, but several imperative factors must be taken into account. Regarding the glycosyl donor, one of the most important factors is the protection of any unsubstituted hydroxy group, in order to obtain selectivity to the chosen acceptor, since its own -OH groups are in most cases also nucleophiles, competing in the reaction and leading to mixtures. One of the few examples where this can be desirable, is the formation of polysaccharides of a single monomer.

This regioselectivity issue has caused the continuous development of efficient protection/deprotection systems, capable of resisting to the reaction conditions, until deprotection is desired[119,120].



Scheme 2. General glycosylation mechanism

Under certain conditions, these protecting groups can also aid in the formation of a desired monomer, due to the neighbouring group effect. This effect is present when an electron rich protecting group is used in the neighbouring position (position 2) to the anomeric position, leading to the stabilization of the formed glycosyl cation by the delocalization of the positive charge, stabilized by resonance (Scheme 3). Several groups have been studied as capable of influencing the result of the glycosylation reaction, and a broad range of effects can be obtained[121,122]. Most of the compounds bearing acyl groups in position 2 lead to the formation of 1,2-*trans* glycosides (Scheme 3), that are stereochemically less hindered, but in the absence of neighbouring group participation, most of the systems generate the α anomer, due to the anomeric effect.



Scheme 3. Mechanistic representation of the neighbouring effect in a glycosylation reaction.

This general preference for the α anomer, the anomeric effect, can be explained by two models, since no consensus exists[123–125]. The first interpretation to emerge, with its roots in the discovery of the anomeric effect[123], is based on the electrostatic interaction of the dipoles formed by the endocyclic oxygen and the polar bond between the exocyclic heteroatom and the anomeric carbon (C1) (Figure 5a), a dipole-dipole interaction that does not exist in the case of an equatorial substituent, and better explains the reverse anomeric effect. The other popular model is based in the electron delocalization from the oxygen lone pairs to the vacant antibonding orbital σ_{CY}* (Figure 5b), a n → σ* hyperconjugative interaction that better explains the observed variations in bond lengths and angles around the anomeric position[124,125]. The aforementioned reverse anomeric effect consists in the preference of an equatorial position, occurring in positively charged anomeric substituents[126]. Additionally, the lone pair from an exocyclic heteroatom can

stabilize a preferred anomeric position, by interaction with the endocyclic C-O pair, giving rise to the exo-anomeric effect[127].

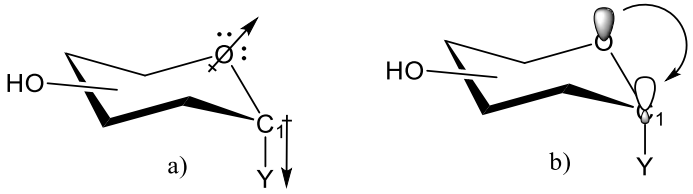


Figure 5. Most common models explaining the anomeric effect. a - electrostatic model; b - hiperconjugation model

Another factor that can influence the outcome of a glycosylation reaction is the solvent. Many studies were also carried out testing this effect, and the most studied solvent for this is acetonitrile, for its largely reported beta orienting effect in several reaction systems. Nonetheless, it is also known that it is not a determinant factor for the outcome of the reaction, and major alfa anomer can be obtained depending on the selection of activators and/or donor/acceptor systems.

Of course, the outcome of a glycoconjugation reaction depends on a careful selection of its two mainly intervening parts, the glycosyl donor and the acceptor. Glycosyl donor halides have been quite used, possibly for its relatively easy preparation and good glycosylation results.[116] Since then, several improvements ensued and a number of diverse glycosyl donor systems were developed, that far surpassed the results of the glycosyl bromide/chloride, as it is the case of glycosyl trichloroacetimidates. Nonetheless, since the preparation of glycosyl iodide and fluoride became more easily accessible, this class of glycosyl donors has remained one of the most utilized. Many other methodological advances have brought forward the expansion to other glycosyl donors specially tailored for an activator, or that suit the specific needs of the acceptor[115]. All these developments aim to provide a solution to one of the greatest issues in carbohydrate chemistry, which is the stereoselectivity in the formation of the glycosidic bond, and the impact of all the effects here discussed are still under debate[117,118].

2.3 Synthesis of alkyl glycosides

The synthesis of alkyl glycosides goes back to the beginning of carbohydrate chemistry and the work of Emil Fisher[115,128]. By refluxing a solution of a saccharide in an alcohol, in the presence of acid, acting as catalyst, provided the first alkyl glycosides, but

quickly a number of glycosylation techniques and/or variations emerged, providing better yields and stereochemical control over the resulting glycosides. These techniques made use of new catalysts and/or glycosyl donors, like glycosyl halides in Koenigs-Knorr[115,129] reaction or the much later developed thioglycosides by Ferrier[130].

Given this, the initial synthesis of medium chain alkyl glycosides (with 8 to 14 carbon atoms comprising the aliphatic chain) relied in acid-catalysed[131] or Koenigs-Knorr type reactions[102]. With the evolution of glycosylation reactions several other methodologies have emerged for the synthesis of alkyl glycosides[132].

At the same time, as our knowledge of biosynthetic pathways evolved, the development of enzymatic catalysis has been of major importance since it allows precise control over the stereoselective formation of glycosidic bonds. Even though this would allow the synthesis of enantiomerically pure glycosides, their *in vitro* application poses some challenges, like the solvent mixture used, that is a compromise between reagent solubility and enzyme activity, or the fact the enzymes need to be produced, isolated and immobilized for their correct use[133]. One of the most successful glycosylation methodologies employs trichloroacetimidates as donors, mainly for the formation of 1,2-*trans*-glycosides[134]. One main advantage of this technique is the fact that it yields glycosides even with weak nucleophilic aglycones.

Although the discussed strategies can be used for a variety of glycoside donors and acceptors, several methodologies were developed, tailored to obtain specific end products or families thereof. One example is the use of glycals when intending to obtain 2-deoxy glycosides[135].

Direct glycosylation making use of glycals relies on a number of activators such as gallium (III) chloride[135] to obtain 2-deoxy glycosides in good yields (>70%) and not only *O*-glycosides but also their *S*- and *N*-analogues. Activators can also be selected according to the desired stereochemistry, for example, when using triphenylphosphane hydrobromide or similar systems, a pronounced α-stereoselectivity is obtained.

There are a number of diversified systems and synthetic pathways leading to the desired products, and in the growing field of carbohydrate chemistry, the number of stereochemically selective strategies is finally coming to pair with the vast chemical space that carbohydrates represent.

3 Alkyl glycosides as antimicrobials

3.1 What is known about alkyl glycosides and where to improve

Previous work by the research group lead to the synthesis of the first compounds of a new family of deoxy glycosides[136,137] and their surfactant properties as well as their antimicrobial potential were studied, and this work came to constituting an important starting point for the work further developed.

The compounds described consist of a 2-deoxy or 2,6-dideoxy sugar moiety, conjugated with an aliphatic alcohol, forming octyl or dodecyl glycosides, easily accessed by employing a glycal as a glycosyl donor and the corresponding saturated alcohols as acceptors, and using triphenylphosphane hydrobromide as a promoter; a simple methodology that yields the alfa anomer as the major product and in reasonably high yields[138].

Then, the properties of these compounds were preliminarily assessed, namely their antimicrobial activity, surfactant activity and cytotoxicity[137]. The results of antimicrobial activity by paper disk diffusion methodology have shown bioactivity towards Gram-positive bacteria and no activity towards Gram-negative bacteria and fungi. The study of its surfactant properties revealed that the aggregation tendency, described as critical micellar concentrations (CMC), and adsorption are promoted by the deoxygenation pattern, but the same overall behavior is observed on the literature data reported for structurally related surfactants. Unfortunately, these compounds also show toxicity towards lymphocytes.

From these results, summarized in table 1 and 2, one compound emerged with unexpected selective antimicrobial activity towards the genus *Bacillus*, and this pronounced activity does not correlate with any difference in adsorption properties between similar compounds, both in the same study and in available literature[107,139]. Hence, the dodecyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside was considered the core molecule and the target of several structural modifications in an attempt to optimize the antimicrobial activity observed, while maintaining the specificity to the genus Bacillus, and also to lower the toxicity of this type of compounds, in order to achieve a suitable therapeutic window.

Table 1. Antimicrobial activity expressed by the diameter of the inhibition zones ± standard deviation (mm) for dodecyl deoxyglycosides.

Compound Name	Dodecyl 2-deoxy-α-D-*arabino*-hexopyranoside	Dodecyl 2-deoxy-β-D-*arabino*-hexopyranoside	Dodecyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside	Dodecyl 2,6-dideoxy-β-L-*arabino*-hexopyranoside
B. cereus	<6.4	10 ± 0	27 ± 5	<6.4
B. subtilis	9 ± 0	10 ± 0	25 ± 3	<6.4
E. faecalis	<6.4	17 ± 2	13 ± 2	<6.4
E. coli	<6.4	<6.4	<6.4	<6.4
L. monocytogenes	10 ± 1	<6.4	12 ± 2	<6.4
P. aeruginosa	<6.4	<6.4	<6.4	<6.4
S. enteritidis	<6.4	<6.4	<6.4	<6.4
S. aureus	<6.4	<6.4	9 ± 1	<6.4
A. niger	<6.4	<6.4	<6.4	<6.4
C. albicans	9 ± 0	10 ± 1	<6.4	<6.4
P. oryzae	<6.4	<6.4	<6.4	<6.4

*Data adapted from Silva et all (2008)[137]

Table 2. Adsorption properties of dodecyl glycosides at the air–aqueous solution interface.

Compound Name	CMC (mol kg^{-1})	γCMC (mNm^{-1})	pC_{20}
Dodecyl 2-deoxy-α-D-*arabino*-hexopyranoside	--	--	--
Dodecyl 2-deoxy-β-D-*arabino*-hexopyranoside	2.2×10^{-5}	32.5	5.1
Dodecyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside	1.1×10^{-5}	28.4	5.7
Dodecyl 2,6-dideoxy-β-L-*arabino*-hexopyranoside	--	--	--
Dodecyl-β-D-*gluco*-hexopyranoside	1.9×10^{-4}	39.1	4.2

*Data adapted from Silva et all (2008)[137]

3.1.1 Analysis of the available literature

As previously referred, alkyl glycosides are known since the end of the nineteenth century, but the available literature is not as extensive as one could expect. Examples of such compounds can be found[107,140–142], and most of them consider their surface activity and adsorption capabilities, but not many of them refer to their biological activities. So, a comprehensive study on the structural features important for their biological activity could not be found. Nonetheless, the relationship between activity and structure with the available reported data was studied in order to trace a profile of which structural features can be of most importance in our lead moleculeand to establish which characteristics of the compounds under study are unique or in compliance with the literature.

Data reported in the literature related to alkyl chains with less than 8 carbon atoms and more than 16 was included only throughout a small number of representative structures, since, in general, their biological properties are worse than between these values and they are out of the range desired for this work. Likewise, other substituents like amines in the sugar moiety were placed only as representatives, since the compounds under study are non-ionic surfactants and any amine residues can become protonated under physiological media, implying a different mode of action[143], and so, not the focus of the study.

Collected data was organized and normalized (Annex 1[139,142–146]). One of the most relevant information obtained from this analysis is a clear relationship between the length of the aliphatic chain and the biological activity. For all the compounds found in the literature, antimicrobial activity raises with length of the aliphatic chain until an optimal range of 11-13 carbon atom length. This is also in accordance to the activity of the simple corresponding alkyl alcohol. Both the α and β anomers display very similar bioactivities, although the β anomer shows better activities toward fungi. The limited modifications on the configuration of the sugar moiety reveled a slight improvement in antimicrobial activity for the mannoside residue. Finally, the protection of hydroxy groups causes an increase in the minimal inhibitory concentration (MIC) values observed.

By comparing what was known about the compound lead and the conclusions obtained by literature analysis, two main differences were observed: firstly, the antimicrobial action of the compound lead was more pronounced against the genus Bacillus, and generally these surfactant compounds present a broad spectrum of activity;

secondly, the bioactivity of the α and β anomers are very similar in all the examples observed, but the β anomer of the lead compound shows no bioactivity.

In the face of such divergences from the traditional profile of biological activity, it is plausible to consider the hypothesis that such compound is exhibiting its antimicrobial activity by a new mechanism of action, other then the surfactant action expected of alkyl glycosides, or at least in addition to it.

3.2 Objectives of the work proposed

Incorporating all the information discussed, the present work was outlined has having two main objectives: 1) the creation of a small library of derivatives to the lead compound, aiming at optimizing bioactivity / toxicity and enabling the structure-activity relationship study; 2) the study of the mode of action for these compounds, despite their surfactant properties.

These objectives were then further developed and the work to be performed subdivided. In order to achieve the first objective, the modification of the starting scaffold, compound **1** (Figure 6), were divided into: i) modifications of the aliphatic portion, the aglycone; ii) modifications on the sugar moiety; iii) modifications of the glycosidic bond. By modifying each feature independently, we could observe the relationship between each structural modification and its impact in antimicrobial and toxicological properties.

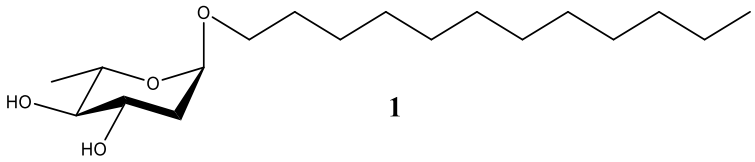


*Figure 6. Lead compound: Dodecyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside (1)*

Regarding the second main objective, in order to study if, apart from a surfactant action, we can observe additional modes of action, several steps were postulated: i) detailed study of the antimicrobial action and its kinetic parameters; ii) the study of the metabolic impact, where the surfactant activity would lead to a non-specific impact and any other putative targets would impact specific metabolic pathways; ii) a genetic approach in order to confirm any target discovered.

Such objectives provided the guidelines for the work here presented.

4 Glycoconjugation towards molecular entities with potential in Alzheimer's disease

Infection is reemerging as a worldwide concern due to the growing bacterial resistance, with the elderly having the higher mortality rates. This population segment is also the most affected one by a number of age-related non-curable diseases[147]. In the context of an European project focusing on the Alzheimer's disease (AD), the collaboration with the partner Biofordrug allowed to evaluate compounds' toxicity with Caco 2 cells and to investigate the synthesis of new glycoconjugates, expected to improve aglycone bioactivity and bioavailability. A brief introduction to the subject and the presentation of the objectives of the work developed are presented below.

4.1 Brief introduction to Alzheimer's disease and available therapy

4.1.1 Alzheimer's disease

Alzheimer's disease is a chronic neurodegenerative disease and the prevailing cause of dementia. It is a slow progressing disease and the absence of perceptive symptoms in the first stages makes it very difficult to detect, only with specialized examination. It's only when symptoms like memory impairment or other cognitive dysfunctions are perceptive that the closest relatives begin to notice changes, but even then they can be mistaken by normal aging.[148–150]

The symptoms for Alzheimer's are very difficult to distinguish in a pre-clinical stage, but upon close examination a sort of global cognitive deficit can be observed, with slight impairments in episodic memory and executive function.[149] At its clinical stages there are clear signs of disease progression, specially associated with the memory, and several biomarkers that can be used to understand its severity and progression. Finally, in the late stages, dementia onsets and even the everyday and most basic functions are impaired.[150,151]

Biologically, AD is characterized by the accumulation of intra- and extra-neuronal β-amyloid (Aβ) as well as intra-neuronal Tau (leading to plaque/tangle formation), factors that drive a cascade of biological processes leading to inflammatory processes and ultimately to neuronal degeneration.[152,153] The factors that cause this accumulation and

ensuing processes are still not fully understood, despite the prolonged efforts that provided insight on many of the biological mechanisms associated with it. This knowledge is essential to the development of new therapeutic options to effectively cure AD.[153]

Presently, the potentially most successful approach, and the major focus of interest, relies in the prevention and successful delay on the most damaging stages of this disease is its detection in a pre-clinical stage, where several precautions can be taken in order to minimize the suffering of the patient and the patient's family.[154,155] For this purpose, several methodologies were applied aiming at finding possible biomarkers in preclinical AD, like structural and functional imaging, and analysis of cerebral fluid amyloid and tau proteins.[155–160] Nevertheless the type of assays necessary to detect these biomarkers are costly and hard to use in a large screening-based manner. Hence, the development of blood-based biomarkers is of great importance for its cost- and time-efficiency and studies have been carried out based on detection of proteins and lipids in the blood, autoantibodies and micro-RNA.[161–167]

It is in this last category that the present research is inserted, but focused in a new and different type of biomarker: metallic ions, namely, copper[168,169].

4.1.2 Current therapeutic approach

Nowadays, after diagnosis of Alzheimer's disease, probably already in the symptomatic stages, the therapeutic intervention can only provide relief of the symptoms, without being able to effectively treat the underlying causes or stopping the evolution of the disease[170].

Most of the available drugs are acetylcholinesterase inhibitors (donepezil, rivastigmine and galantamine) and one N-methyl-D-aspartic acid (NMDA) and glutamate receptors antagonist (memantine)[171]. The latter, being the last approved drug, goes back to 2003, and although some new drugs with diverse targets reached clinical trials, many were discontinued due to safety and/or effectiveness concerns[172].

One of the issues for AD therapeutic development, is that the clinical trials are performed with patients with already advanced pathophysiological signs of the disease, a factor impairing the true assessment of potential drug effectiveness and success rate[172].

Therefore, the development of reliable early detection tools for AD and effective biomarkers to evaluate the progression of the disease are still a primary necessity.

Nonetheless, as currently available drugs fail to alter the primary pathological processes that underlie AD, new therapeutic agents are emerging, targeting Aβ production (namely γ- and β-secretases)[173–175], Aβ clearance (Aβ-degrading proteases)[176,177], protein aggregation (specially Tau)[178] and neuroinflammation (microglia modulators)[179,180].

4.1.3 The role of copper ions in AD

The link between metals and AD or other neurodegenerative diseases is becoming better understood and it is known that they play an essential role in protein aggregation, making them possible biomarkers.[181,182] Copper (II) and zinc (II) ions are indicated as the best candidates for the formation of the first fibrils in protein aggregation, which becomes then an autocatalytic process. Thus, once a certain concentration of free ions in the blood is detected, there is a corresponding probability of these aggregates to be formed in the brain, the beginning of AD.[183,184] Even though research in the role of these ions in AD is quite new, the connection is already indisputable.[185]

Given this association, the possible application of metal chelating compounds as an important early-Alzheimer's biomarker or even as a therapeutic approach, is of growing interest[186,187].

4.1.4 ABC transporters and AD

ATP-binding cassette (ABC) transporters are a superfamily of active transporter systems formed by multiple subunits, one transmembrane domain and an ATP-binding cassette domain. They perform various functions and can be importers or exporters.

The relation between this type of transporters and AD has been discovered in the last decade and only recently these transporters are looked at has a viable therapeutic target. But, nonetheless, a relationship between various subfamilies of ABC transporters and AD has been established, mainly for ABCA, ABCB and ABCG.[188–191]

P-glycoprotein (P-gp) is an ABC transporter (also called ABCB1) and has been correlated with the rate of accumulation of amyloid protein in the brain, becoming a possible therapeutic target for the treatment of AD. It is highly present in the blood-brain barrier (BBB) and several studies indicate that an overexpression or "over-activation" of

this protein is correlated with decreased Aβ brain load.[192–196] Because of this, the investigation of improved pharmacokinetics in inducers of P-gp has become a secondary goal of the work performed.

An important note is that P-gp is not a transporter system exclusive to the BBB, it is greatly expressed in the intestinal epithelium, liver and kidney, and its overexpression in several cancer types is associated to multidrug-resistance, all of which has an important impact in the potential applications of modulator compounds.

4.1.5 Improving with carbohydrate conjugation

The incorporation of a sugar residue by a glycosidic bond in bioactive molecules is a commonly employed strategy to improve compound solubility, bioavailability and, in general, the pharmacokinetic profile[197,198], as discussed before.

Nonetheless, in the specific case of metal chelating molecules, this approach is of increased value, since one of the main advantages of such glycosylated compounds is the masking of their chelating function, leading to the prevention of side effects due to systemic chelation. Hence, such compounds can function as a prodrug, since the hydrolysis by glycosidases will increase the chelating capacity only in targeted tissues.[187,199]

Additionally, a glycoconjugate approach in order to enhance the selectivity towards the central nervous system (CNS) has been successful in other cases, once the BBB has a high density of hexose transporters, named GLUTs, responsible for the intake of glucose to the brain, and some glycoconjugates have shown an equally positive brain uptake.[199–201]

Chapter II – Results and Discussion

5 Alkyl glycosides as antimicrobials

5.1 Synthesis of antimicrobial alkyl glycosides

Alkyl glycosides as antimicrobials From the past study previously discussed, several conclusions provided insights into the advantageous structural features for this family of compounds. For example, the alkyl 2-deoxy-D-*arabino*-hexopyranosides exhibited no significant antimicrobial properties, except with *Enterococcus faecalis* that was susceptible to the beta anomers, and so, 2,6-dideoxygenation patter was deemed important to bioactivity.

Regarding the alkyl 2,6-dideoxy-L-*arabino*-hexopyranosides, the octyl glycosides showed an unremarkable broad spectrum of inhibition, but regarding the dodecyl glycosides, interesting and striking differences arise. The dodecyl 2,6-dideoxy-β-L-*arabino*-hexopyranoside showed no biological activity to any of the species tested, while the dodecyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside exhibited a marked antimicrobial activity to both *Bacillus* species tested (MIC = 7.8 μg mL^{-1}), rivalling the antimicrobial potency of the control drug, chloramphenicol (MIC = 3.1 μg mL^{-1} and 6.3 μg mL^{-1}, against *B. subtillis* and *B. cereus*, respectively)[137].

Additionally, some activity regarding *E. faecalis* and *Listeria monocytogenes* was observed, but not as strikingly different from most of the compounds used in the study. Actually, *E. faecalis* sp. appears to be quite susceptible to this type of compounds since, looking to the paper disk screening, 75% of the compounds demonstrated at least a small inhibition diameter. This is indicative that it is especially susceptible to the surfactant capacity of these compounds.

As referred before, merging this information with the conclusions obtained from the literature examples (section 3.1.1), the antibacterial results observed were surprising, not only due to the singular activity towards *Bacillus* spp. of one compound against very similar analogues, but also by the outstanding difference of bioactivity between the α and β anomers. Such observations do not fit the established bioactivity profile for surface active compounds reported in the literature.

This apparent antimicrobial specificity to *Bacillus* species, and α/β disparity instigated the further study of this compound and it was considered the scaffold structure for further studies (Figure 6).

5.1.1 Defining a synthetic strategy

Regarding further compound development, the need of developing compounds that would allow us not only to overcome the observed toxicity but mainly that could help us to establish a mode of action for this type of compounds was the driving force. So, a library of analogues to this compound was generated, attempting to provide insight into the essential molecular characteristics for this profile of bioactivity.

The target glycosides were designed intending to explore the importance of the aglycone in the bioactivity, and if a direct relationship between the surface activity and the antibacterial properties could be observed. So, maintaining the sugar moiety, a number of aglycones were conjugated.

Firstly, a range of glycosides comprising saturated aglycones ranging from 10 to 13 carbon atoms were synthesized, in order to establish if a linearity in the relation between chain length and antibacterial activity exists, as it could be expected based on the literature information about the bioactivity of the saturated alcohols[98,202].

Also, modifications to the nature and type of packing of the aglycone were attempted by employing a highly fluorinated saturated aglycone. By introducing this modification, the amphiphilic molecular character is altered by reducing the lipophilicity of the aliphatic chain. At the same time, an opposing modification was performed, by testing the behaviour with a branched saturated chain. This modification can drastically increase the aliphatic portion, altering profoundly the hydrophilic head – lipophilic tail proportion. Such structural changes modify the packing structure of the lipids and may affect the type of interaction with biological membranes.

Regarding the glycon (glycosyl group) of this type of molecules, the information gathered from the literature and our previous studies encouraged modifications of the 2-deoxy position, once the 6-deoxy position was regarded as pivotal to the biological activity observed.

So, in an alignment of interests, a derivative capable of being radioactively labelled was synthesized, by introduction of a halogen atom in position 2. The reasons driving a possibility of radioactive labelling were related to the fact that labelling this type of compounds with fluorophores or other types of labelling techniques would, theoretically, profoundly modify their amphiphilic character. In order to preserve the structural features of the lead compound as much as possible, a 2,6-dideoxy-2-iodo-

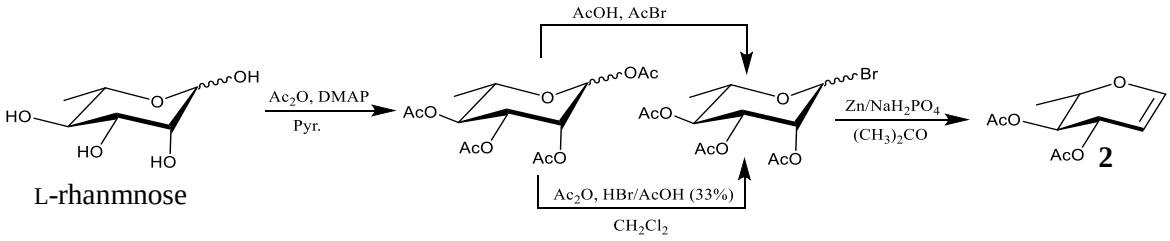
glycoside was prepared, since a radioactive iodine isotope (^{125}I or ^{131}I) can be easily prepared from the "cold" form, by nucleophilic exchange, where halogen-halogen exchange happens in solution[203].

5.1.2 Synthetic effort and results obtained

As stated above, the initial modifications to the core compound **1** (Figure 6) were performed focusing on the aliphatic portion, aiming to understand the impact of the chain length in compound activity, and to check if the activity profile follows the expected pattern, according to the literature.

In order to perform the synthesis of these compounds, the glycosyl donor had to be prepared as well. Since the donor is a glycal, more specifically the 6-deoxy-3,4-di-*O*-acetyl-L-glucal (**2**) (systematic name: 3,4-di-*O*-acetyl-1,5-anhydro-2,6-dideoxy-L-*arabino*-hex-1-enitol), synthetic strategies were tested using L-rhamnose as a starting material, one of the few natural sugars belonging to the L-series.

Several techniques are available from the literature[116,204] for the preparation of glycals. In scheme 4 is depicted the reaction pathway carried out via an intermediate glycosyl bromide. However, reaction optimization was required to improve compound isolated yield.

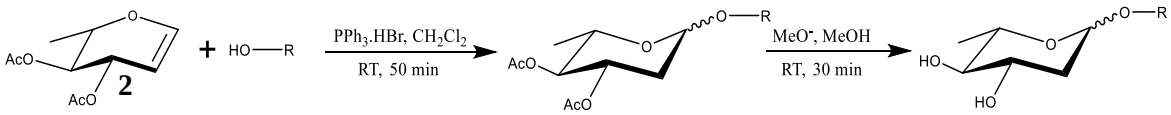


Scheme 4. Glycal synthesis via glycosyl bromide formation.

The first reaction conditions tested used hydrobromic acid in acetic acid solution, but this methodology to form the intermediary bromide was not very successful, as revealed by the average bromide final yield of 39 %. Using the method applying acetyl bromide for the *in-situ* formation of hydrobromic acid, the results obtained were much more reproducible and the yield increased to 70%.

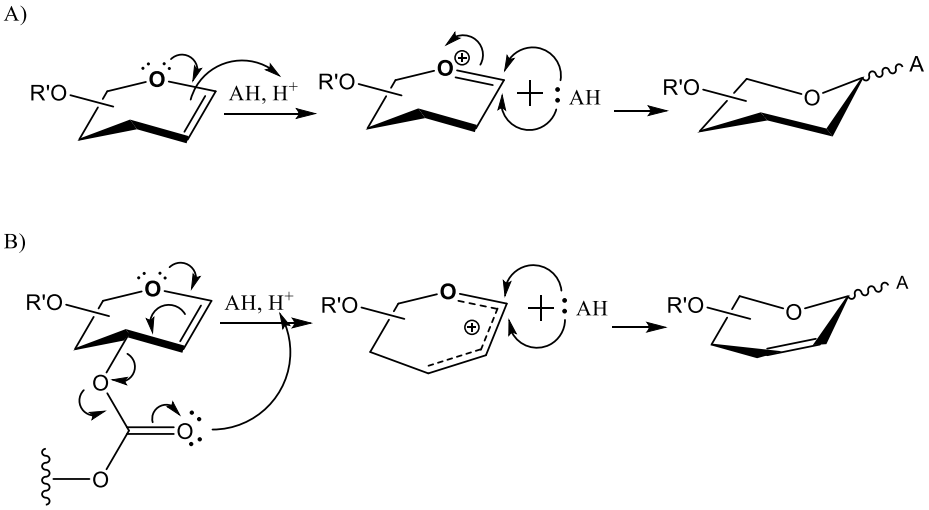
After obtaining the glycosyl donor, the next step consists in the conjugation which was performed by following the known methodology already highly studied in the

research group. A suitable glycal reacts with the alcohol in the presence of triphenylphosphane hydrobromide (TPHB) as shown below (Scheme 5).[137,205]



Scheme 5. Schematic representation of the glycoconjugation reaction.

When using glycals as starting materials, glycoconjugation by acid catalysis (Scheme 6A), as it is the case, can also give rise to secondary products due to Ferrier rearrangement, upon the formation of an allyloxycarbenium ion (Scheme 6B).

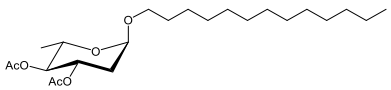
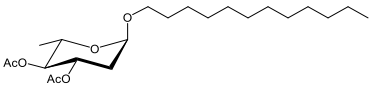
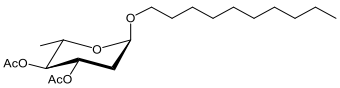
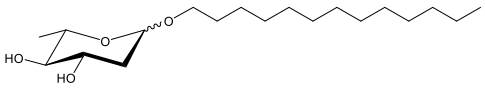
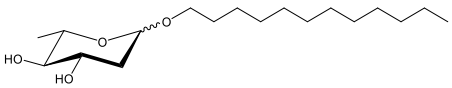
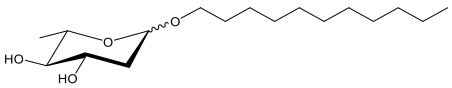
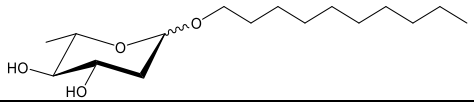


Scheme 6. General mechanism for acid-catalyzed glycoconjugation reaction and the formation of Ferrier rearrangement products.

Which of these reactions is more predominant in the glycoconjugation procedure can be controlled by the choice of catalyst, hence our catalyst of choice for obtaining 2-deoxy glycosides was TPHB, that is known to prevent Ferrier rearrangement by promoting the protonation of the softer C-2 position[138,206]. Additionally, the choice of catalyst was also due to the fact that it gives rise to the α anomer as the major product, which is the most desired anomer, since in the preliminary study only the α anomers have shown significant bioactivity (section 3.1 & 4.1).

The two-step methodology applied involved the purification of the intermediary acetylated compounds (yields for the major intermediary compounds in table 1), but further optimization to a one-pot methodology (section 9.1.4) gave rise to the best yields for all compounds. Yields and stereoselectivity of the one-pot, glycal reaction with saturated alcohols with chains of different lengths are summarized in table 3.

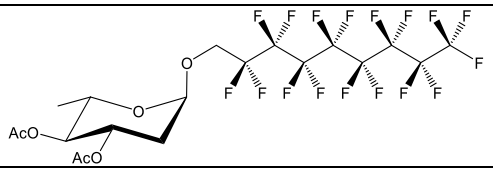
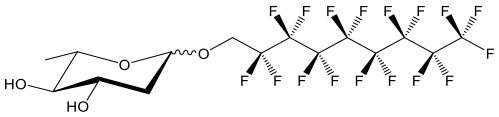
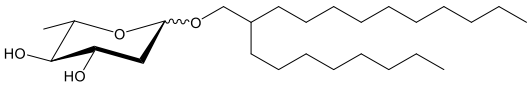
Table 3. Yield of alkyl glycosides with different alkyl chain length

Compound Name	Compound No.	Structure	Yield
Tridecyl 3,4-di-*O*-acetyl-2,6-dideoxy-α-L-*arabino*-hexopyranoside	**3**		57 %
Dodecyl 3,4-di-*O*-acetyl-2,6-dideoxy-α-L-*arabino*-hexopyranoside	**4**		59 %
Decyl 3,4-di-*O*-acetyl-2,6-dideoxy-α-L-*arabino*-hexopyranoside	**5**		61 %
Tridecyl 2,6-dideoxy-L-*arabino*-hexopyranoside	α: **6** β: **7**		α: 69 % β: 23 %
Dodecyl 2,6-dideoxy-L-*arabino*-hexopyranoside	α: **1** β: **8**		α: 67 % β: 14 %
Undecyl 2,6-dideoxy-L-*arabino*-hexopyranoside	α: **9** β: **10**		α: 56 % β: 13 %
Decyl 2,6-dideoxy-L-*arabino*-hexopyranoside	α: **11** β: **12**		α: 71 % β: 15 %

The same methodology was employed to obtain two other derivatives, where the nature of the aliphatic chain was modified. The first derivatives synthesized (**9,10**) contained a highly fluorinated chain, aiming at understanding the impact in bioactivity of a reduction in the lipophilicity of the aliphatic portion, maintaining nonetheless the chemical nature of the glycoside, since fluoride is a well-known bioisostere to hydrogen atoms[207]. A branched aliphatic chain aglycone was also used, in order to evaluate the impact of increasing lipophilicity, resulting in the compounds **11** and **12**.

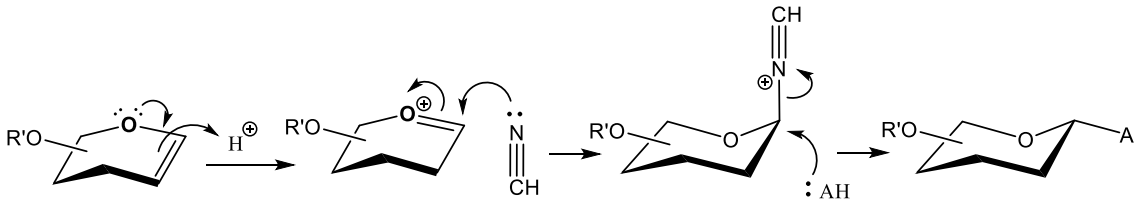
Also, since the 2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-nonadecafluorodecanol presented poor solubility in dichloromethane, for the reaction solvent a mixture of dichloromethane and acetonitrile (1:1) was used instead. The results are presented in table 4.

Table 4. Yield of glycosides embodying a fluorinated and a branched alkyl chain

Compound Name	Compound No.	Structure	Yield
2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-Nonadecafluorodecyl 3,4-di-*O*-acetyl-2,6-dideoxy-L-*arabino*-hexopyranoside	**13**		50%
2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-Nonadecafluorodecyl 2,6-dideoxy-L-*arabino*-hexopyranoside	α: **14** β: **15**		α: 59 % β: 17 %
2-octyldodecyl 2,6-dideoxy-L-*arabino*-hexopyranoside	α: **16** β: **17**		α: 33 % β: 19 %

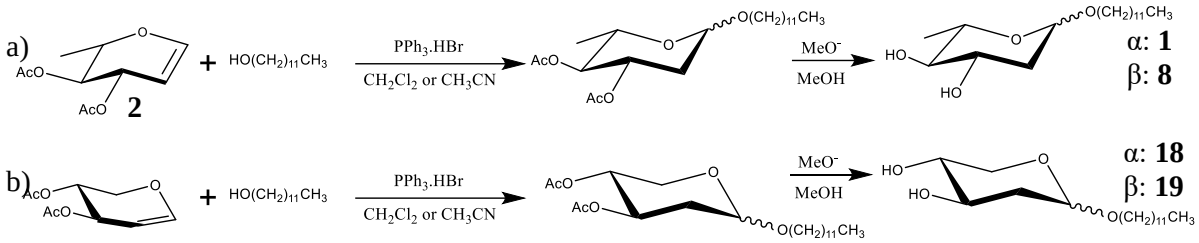
5.1.2.1 Impact of the solvent in α:β ratio

As referred before (section 2.2) the choice of solvent in a glycosylation reaction can also have an important impact in the reaction products, namely the α/β ratio. When employing acetonitrile as a solvent, there is the possibility of it participating in the reaction, following the mechanism represented in the following scheme, leading to the formation of the β anomer as the major product, instead of the α[125,208].



Scheme 7. General mechanism of the role of acetonitrile in leading to the major formation of β anomer.

Since there was the need of using both dichloromethane and acetonitrile as solvents, for example in the synthesis of compound **13**, their impact in this reactional system was assessed. In order to maintain the reaction conditions as similar as possible for the synthesis of dodecyl 2,6-dideoxy-L-*arabino*-hexopyranosides (**1,8**) and dodecyl 2-deoxy-D-*threo*-pentopyranosides (**18,19**), the procedures were performed in parallel, in both solvents, according to scheme 8.



Scheme 8. Reactional system for the study of the impact of solvent in α:β ratio

The analysis of reaction mixtures by ^{1}H NMR spectrometry was performed to detect, in the most unaltered state possible, the proportion of reaction products formed (Figure 7 and Annex 2).

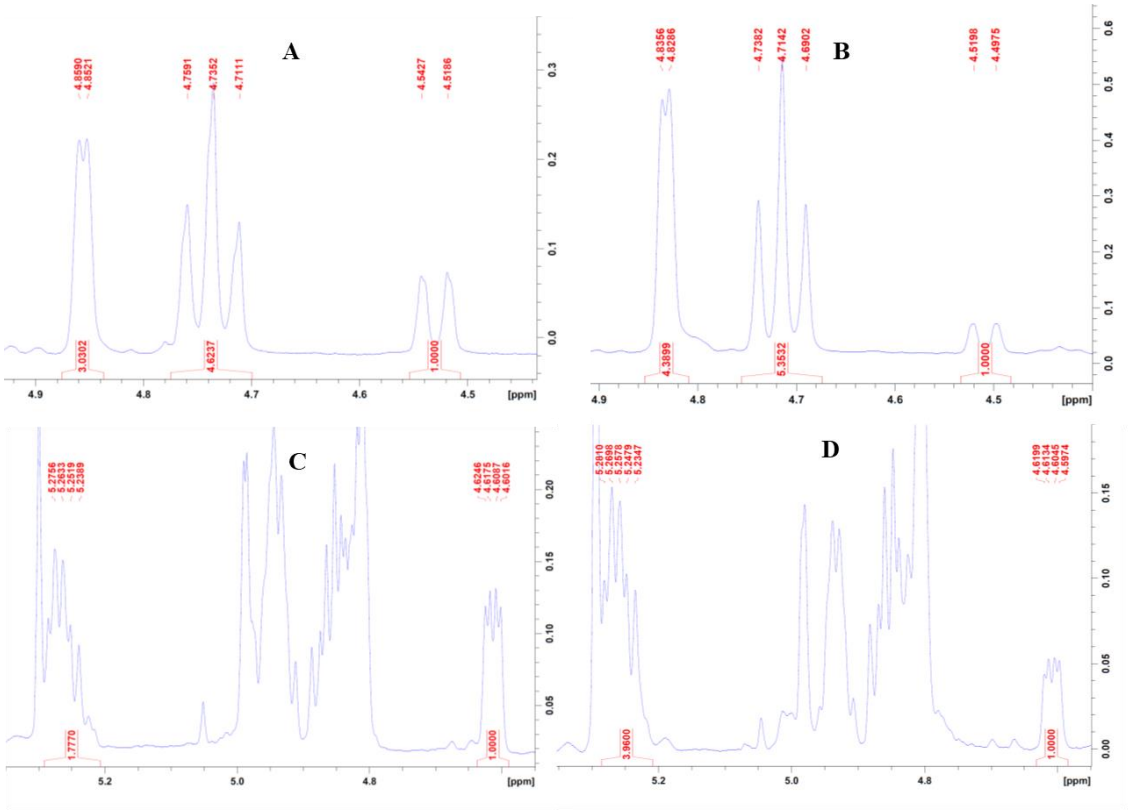


Figure 7. NMR comparison of reactional mixtures and corresponding α:β ratios. A – NMR from reactional system a, in CH_2Cl_2; B – NMR from reactional system a, in CH_3CN; C – NMR from reactional system b, in CH_2Cl_2; D – NMR from reactional system c, in CH_3CN

In order to observe the corresponding ratios α and β ratios, for the reactional system a (scheme 6a; Figure 7 A and B), the integration of peaks corresponding to the anomeric H-1 were used, with H-1α appearing around 4.85 ppm and H-1β around 4.51 ppm. For reactional system b (Scheme 6b; Figure 7 C and D), the integrations used were of peaks H-3α appearing around 5.25 ppm and H-1β around 4.60 ppm.

It was found that there was a higher proportion of the α anomer in both solvents, but with significant difference between the ratios in different solvents, in accordance to the reported[137].

5.1.2.2 Microwave-assisted synthesis

Microwave-assisted synthetic methodologies can benefit numerous reaction systems, since microwave-heating is known to greatly increase reaction kinetics, while also displaying specific and non-thermal microwave effects, whose existence is still widely debated[209]. Nonetheless, microwave-assisted methodologies seem to have a clear advantage over traditional heating methodologies[210], and with additional benefits to specific reactional systems, our synthetic methodologies were adapted to explore their potential optimization under microwave-assisted reactional conditions (Table 5).

Table 5. Comparing yields and reaction times of microwave-assisted synthesis with the conventional method

Compound	Traditional		Microwave-assisted	
	Yield	Time (min)	Yield	Time (min)
1	67 %	50	63 %	10
8	14 %		24 %	
11	71 %	40	56 %	10
12	15 %		18 %	
14	59 %	180	55 %	30
15	17 %		24 %	

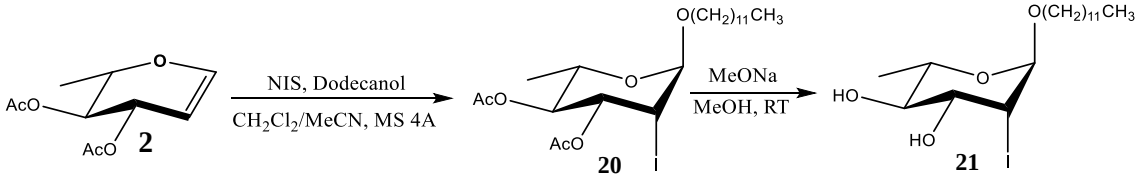
As shown, the reaction times of the microwave-assisted synthesis were significantly lower, however, yields obtained were lower compared to those obtained by the traditional methodology due to an increase in the formation of side products, in particular Ferrier rearrangement products. In the case of compounds **14**/**15**, the least reactive by the traditional methodology, there was a slight increase in overall yield, but due to an increased formation of the β anomer (**15**). This increase of β anomer formation was registered for all the compounds, causing a variation in the α:β ratio and, since the alpha anomer is the compound of interest, microwave-assisted synthesis methodologies proved to be less appropriate than traditional methods.

5.1.2.3 Radioactive isotope labelling

Other modifications to the aliphatic chain were performed, namely having a terminal functional group (compound **G9**, Figure 18), capable of further functionalization. The idea behind this development was the possible functionalization with a fluorophore, as a mechanism of tracing and visualizing the compound in the biological media. However, preliminary results showed that any modification in the terminal position of the aliphatic portion of these compounds would lead to a complete loss of measurable bioactivity (Table 5 and/or Figure 18). Although unfortunate, such results could be expected since bioactivity of this type of compounds relies on their amphiphilic character and such a variation would hinder its capacity to access its potential biological target[211].

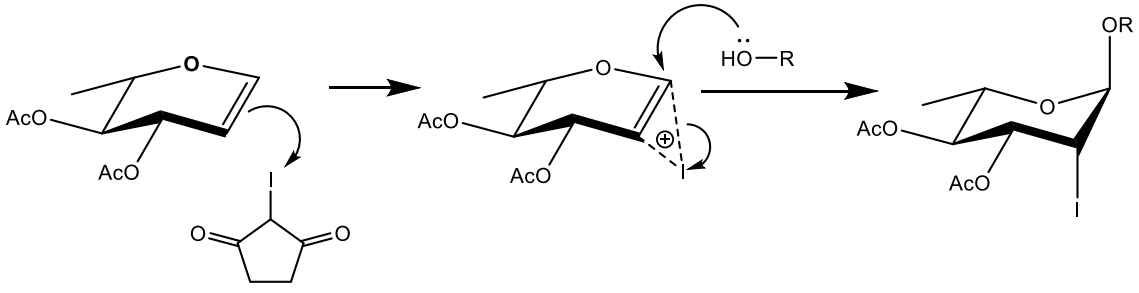
Given these results, an alternative methodology to label the compounds was proposed, one that would alter as little as possible the aliphatic character. A possible solution is radioactive labelling, and to test this hypothesis, an iodine radioactive isotope was planned.

The synthesis of compound **1** iodinated analog was performed with the same glycosyl donor and acceptor but employing *N*-iodosuccinimide (NIS) has a catalyst for the glycoconjugation[212,213] (Scheme 7)



*Scheme 9. Reaction scheme for the synthesis of dodecyl 2,6-dideoxy-2-iodo-α-L-*arabino*-hexopyranoside*

Employing this methodology, compound **21** (Dodecyl 2,6-dideoxy-2-iodo-α-L-*arabino*-hexopyranoside) was obtained with 61 % yield, and only the alfa anomer of this compound was isolated, as expected by the mechanism of this reaction (Scheme 8).



Scheme 10. Mechanism for the glycosylation reaction catalysed by NIS.

5.2 Establishing a compound library and its antimicrobial efficacy

The synthesis previously described was part of the development of alkyl L-*arabino*-hexopyranosides, but the synthetic effort of the entire research group produced other families of compounds as well, such as alkyl D-*arabino*-hexopyranosides, D-*threo*- and L-*threo*- pentopyranosides. The summary of the most relevant compounds obtained, presented in figure 18, provides an overview of the structural diversity of the compounds synthesized and the functionalization capabilities of such structures. Alongside the development of such compounds, the determination of their antimicrobial capabilities allowed for a rational design of further structures and provided guidance to the work performed.

A compounds antimicrobial activity is assessed *via* the determination of the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC). While MIC values indicate the lowest concentration of antimicrobial agent that inhibits observable bacterial growth, MBC values indicate the lowest concentration necessary to kill the bacteria, which makes them two complementary results, essential to the correct determination of the antimicrobial potential associated to any compound.

When a bacterial culture is submitted to an antibacterial agent and no bacterial growth is observable, it is not possible to know if the bacterial growth was simply inhibited by the presence of this compound (bacteriostatic effect) or if the bacteria were in fact rendered non-viable or killed (bactericidal effect). That is the reason why, after the MIC determination, the bacterial culture submitted to the compounds' action is then transferred to growth media devoid of antimicrobial compound, where any surviving CFUs (Colony Forming Units) will resume growth. After 16-24 hours, the MBC results are obtained.

The determination of bacterial susceptibility in previous studies was performed in using solid media agar dilution[136] as a methodology, and for experimental reasons, the microdilution methodologies would be much more favorable for all the studies planned. So, a comparison between both techniques was done to validate the results obtained. These methods were applied according to the standards provided by the Clinical and Laboratory Standards Institute[214]. At the same time, using microdilution methods provided the possibility of obtaining high resolution microbial growth curves, that allow

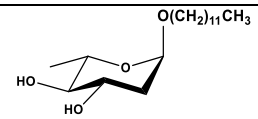
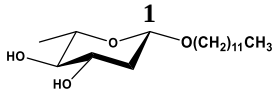
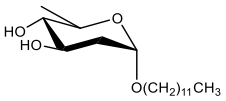
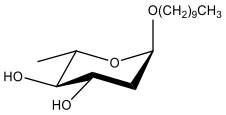
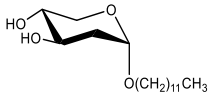
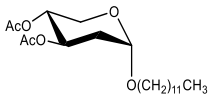
not only the determination of the susceptibility to the compounds, as well as the observation of the impact on bacterial growth and its evolution over time, including at sub-inhibitory concentrations.

Based in the previous results[137], the bacterial strains for the antimicrobial susceptibility determination were selected. Firstly, there was no bioactivity detected in Gram-negative bacteria, fungi and yeast cells, so, they were not focused in the assays performed. Regarding the Gram-positive bacteria, as previous results show bioactivity specially towards *Bacillus* genus, several strains were selected, in order to observe if this activity was the same in amongst other strains of Bacillus, so, *B. cereus* ATCC 14579, *B. cereus* ATCC 11778 and *B. subtilis* ATCC 6633 were selected.

In addition to the *Bacillus* strains referred above, two reference bacterial species were added: *Staphylococcus aureus* ATCC 29213 strain, a Gram-positive bacteria, and *Escherichia coli* ATCC 8739 strain, a Gram-negative bacteria, and both widely used as representatives of their respective Gram classification. Also, the lack of bioactivity of these alkyl glycosides to S. aureus has become a hallmark in the bioactivity profile, since it was unexpected according to the literature, as discussed before.

Results for the susceptibility assay in the bacterial strains described as well as the results for the comparison between both methodologies used are summarized in the following table.

Table 6. Comparison of the results obtained for susceptibility determination.*

Compound	MIC (µg/mL)				
	B. cereus ATCC 14579	*B. cereus* ATCC 11778	*B. subtilis* ATCC 6633	*S. aureus* ATCC 29213	*E. coli* ATCC 8739
 **1**	16(8)	16(8)	16(8)	>128 (>128)	>128 (>128)
 **8**	128(128)	nd	nd	nd	nd
 **G1**	16(16)	16(16)	16(16)	>128 (>128)	>128 (>128)
 **11**	32(32)	nd	nd	nd	nd
 **12**	128(128)	nd	nd	nd	nd
 **18**	16(8)	16(8)	16(8)	>128 (>128)	>128 (>128)
 **G2**	>128 (>128)	>128 (>128)	>128 (>128)	>128 (>128)	>128 (>128)

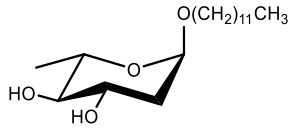
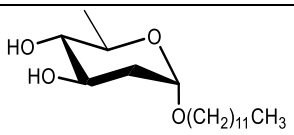
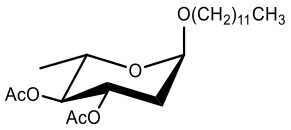
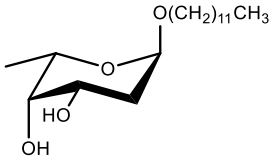
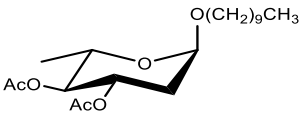
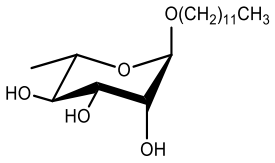
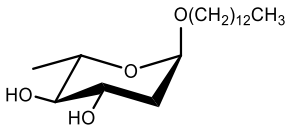
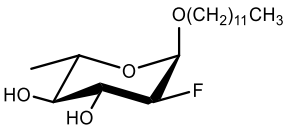
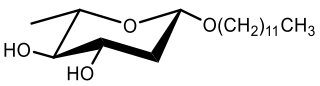
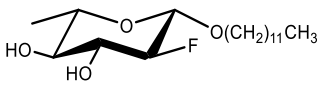
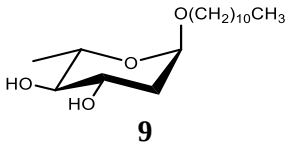
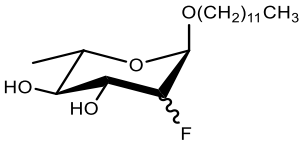
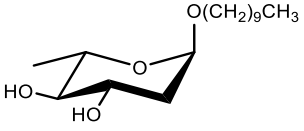
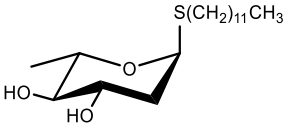
*Values inside parentheses refer to solid media assays results and outside to microdilution liquid media assays

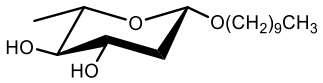
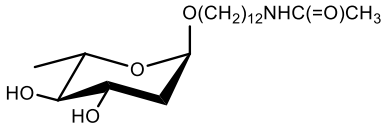
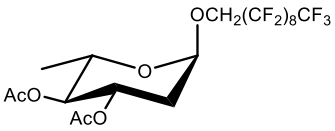
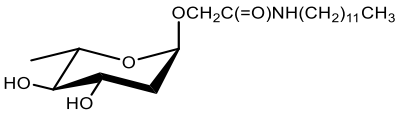
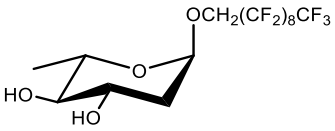
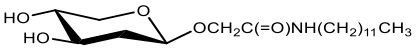
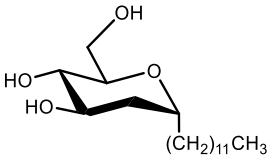
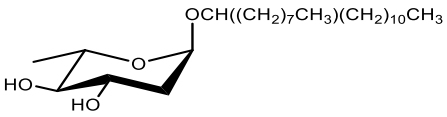
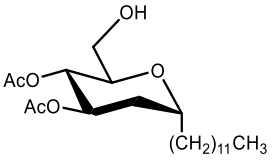
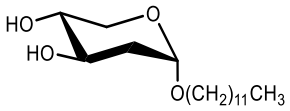
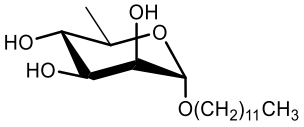
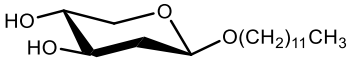
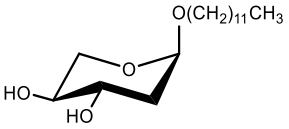
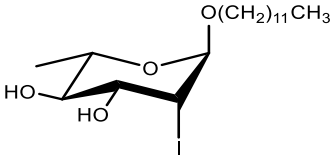
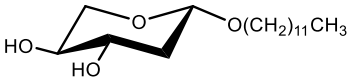
Both methodologies provided very similar results and the microdilution methodology was used for the remainder of the work developed and the results were comparable to those obtained using solid media.

Additionally, the microdilution methodology, by using an automated plate reader, provides additional information to the determination of MIC values, providing bacterial growth curves in all the conditions tested (Annex 3). This allows for the complementary acquisition of the profile of bacterial response to the action of all compounds tested. It was this additional information that allowed the observation of the unexpected effects of compound **14**, subject discussed in section 8.2 (Annex 4).

In order to simplify the screening technique for the remainder of the compounds tested, only a single bacterial strain was selected. The strain *B. cereus* ATCC 14579 was selected as being representative of the genus *Bacillus*, since its genome has been completely sequenced and extensively studied[215–218], a fact that will help during the search for the mechanism of action, where this will also be the selected strain. Also, compound **1** was also used as inter-experimental control once it has shown the most promising bioactivity in the previous studies as well as in the present screening studies. The results are summarized in table 7.

Table 7. Antimicrobial activity over *B. cereus* ATCC 14579 determined for all the compounds synthesized by the research group*

Compound structure and No.	MIC	MBC	Compound structure and No.	MIC	MBC
 **1**	16	16	 **G1**	16	16
 **4**	>128	>128	 **G3**	32	32
 **5**	>128	>128	 **G4**	32	32
 **6**	64	64	 **G5**	16	16
 **8**	128	128	 **G6**	>128	>128
 **9**	16-32	32	 **G7**	16	16
 **11**	32	32	 **G8**	128	128

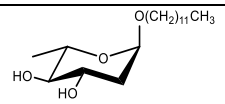
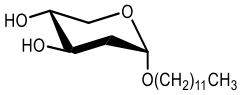
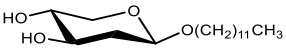
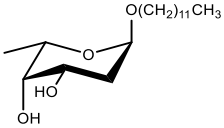
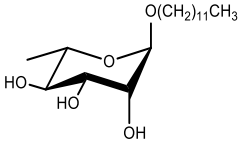
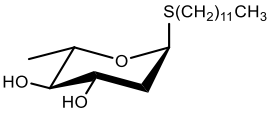
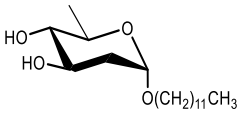
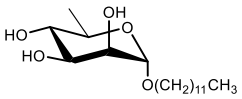
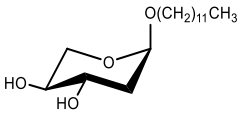
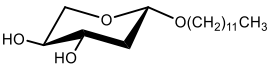
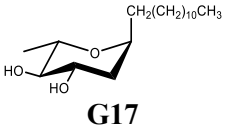
 **12**	128	128	 **G9**	>128	>128
 **13**	>128	>128	 **G10**	64	128
 **14**	>128	>128	 **G11**	64	128
 **15**	>128	>128	 **G12**	128	128
 **16**	>128	>128	 **G13**	>128	>128
 **18**	16-32	32	 **G14**	32	32
 **19**	16-32	32	 **G15**	32	32
 **21**	16	16	 **G16**	32	32

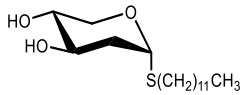
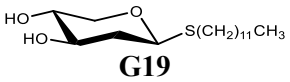
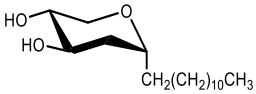
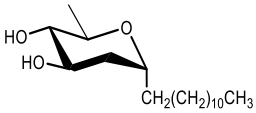
* Compounds designated as G were synthesized by other members of our research group. Antimicrobial activity expressed by MIC (µg/mL) and MBC (µg/mL), determined by microdilution methods (section 9.3.2)

Since *B. anthracis* is one of the most important pathogens in the *Bacillus* genus, especially since its weaponization and use in bioterrorism[219], a selected group of compounds was tested against B. anthracis strains, but the study of this species is restricted to level 3 biosafety laboratories, which I could not operate, and so such studies were performed at the National Institute of Health Instituto Dr. Ricardo Jorge. Results

obtained for 3 different B. anthracis strains are presented in table 8 and were already published[205].

Table 8. Antibacterial activity expressed in MIC (µg/mL)

Compounds	*B. anthracis sterne*	*B. anthracis pathogenic*	*B. anthracis ovine*	*E. faecalis*	*B. cereus*
 **1**	8	8	8	>16	8
 **18**	8	16	16	>16	16
 **19**	8	8	16	16	16
 **G3**	8	8	8	>16	8
 **G4**	8	8	16	16	n.d.
 **G8**	8	16	16	>16	16
 **G1**	16	16	16	16	8
 **G14**	8	8	>16	>16	16
 **G15**	8	16	16	16	n.d
 **G16**	8	8	16	16	n.d.
 **G17**	8	16	16	>16	8

 **G18**	16	8	16	16	8
 **G19**	8	16	16	>16	16
 **G20**	8	8	8	16	8
 **G21**	8	8	8	>16	8

[a]Agar dilution method following CLSI guidelines, compound concentration ranging from 8 to 16 µg/mL.

5.2.1 Evaluation of the cytotoxicity of alkyl deoxy glycosides

Evaluation of compound biological activity is as relevant as the determination of its cytotoxic effects for applications in human health. This is especially true to the studied family of compounds, since their surfactant properties are associated to promiscuous interaction to all kinds of biological membranes, including eukaryotic cell membranes. If similar levels of bioactivity are detected to both prokaryotic and eukaryotic organisms, this would be an indicator that only the surface activity is responsible for the effects observed, and possible applications of this type of compounds would be limited to cleaning and disinfection formulations[108,220,221].

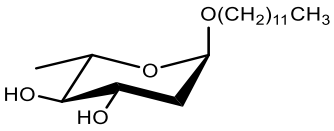
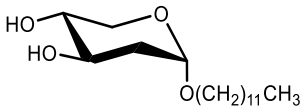
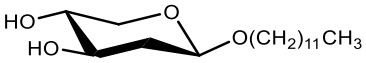
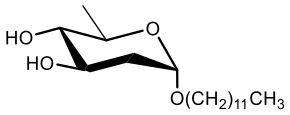
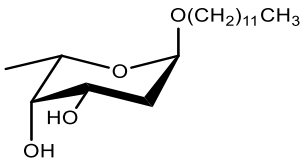
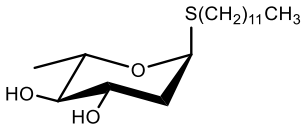
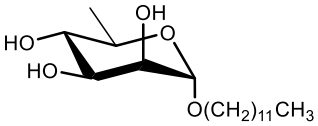
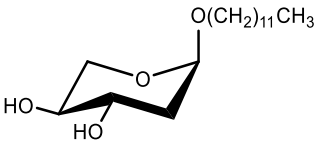
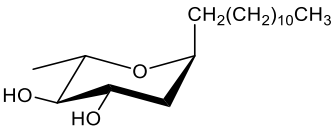
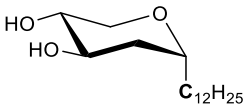
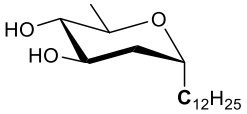
Evaluating the toxicity of a selected group of compounds was possible by applying the MTT assay, a colorimetric assay allowing the visualization of cellular metabolic activity with and without exposure to any agents. In this methodology, the desired cellular culture is exposed to the action of the compounds in study, and after, a tetrazolium dye, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in this case, is added. This dye, in the presence of metabolic active cells, can be reduced by oxidoreductase enzymes dependent of NADH and/or NADPH. This dependence means that only metabolic active (NADH/NADPH producing) cells, will reduce MTT to formazan, a purple, insoluble dye. This dye is accumulating in live cells, and upon cell lysis, it can be spectroscopically quantified, this way allowing the direct correlation between MTT absorption values and cellular vitality.

For this assay, Caco2 cells were selected as a model for human tissue, since they are human epithelial cells derived from a colon carcinoma. The cytotoxicity was

evaluated in Caco2 cells grown in supplemented DMEM high glucose media, in a humidified incubator at 37 °C with a 5 % CO_2 atmosphere. After the established incubation time with the desired compounds, MTT (0.5 mg/mL) was added to the cell suspension, leading to the formation of formazan crystals.

The results were obtained by measuring the absorbance of said crystals in a solution of DMSO/EtOH (1:1), in a microplate reader. The graphical representation of the variation of cell viability with the compounds concentration (Figure 8), allowed the determination of the IC_{50} values (Concentration at which a 50% inhibition in a biological process is observed *in vitro*), and the results are shown in the following table (Table 9).

Table 9. Cytotoxicity over Caco 2 cells determined using the MTT method.

Compound Structure	Nº	IC_{50} (µM [µg/mL])	MIC (µg/mL)
	**1**	59.0 [18.7]	16
	**18**	142.1 [43.0]	16-32
	**19**	87.9 [26.6]	16-32
	**G1**	58.3 [18.5]	16
	**G3**	56.3 [17.8]	32
	**G8**	58.5 [19.5]	128
	**G14**	62.0 [20.7]	32
	**G15**	115.9 [35.0]	32
	**G17**	121.8 [36.6]	8*
	**G20**	103.0 [29.5]	8*
	**G21**	139.0 [41.8]	8*

*Results obtained by dilution in solid media at National Institute of Health Dr. Ricardo Jorge

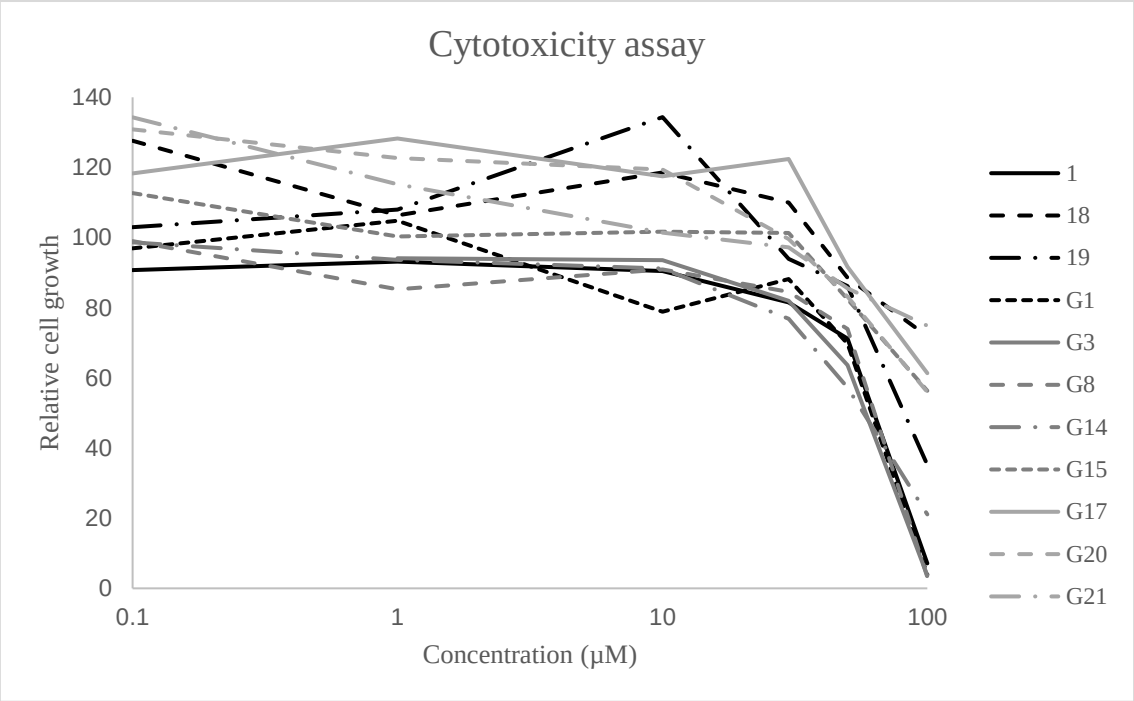


Figure 8. Graphical representation of the cytotoxicity assay of a group of compounds selected according to the results obtained for bioactivity in B. anthracis spp.

Unfortunately, as we can see, these compounds present similar values of MIC and IC_{50}, meaning that these compounds are toxic to human cells, and that the structures synthesized did not successfully presented an answer to this issue, nonetheless the C-glycosides are, generally, the least toxic compounds.

Also, no obvious correlation between antimicrobial activity and toxicity was found, hence the mechanism of these two biological effects could be unrelated.

5.2.2 Surface disinfection and antibacterial activity

Several patented applications of similar compounds (alkyl glycosides) show that the emulsifying and surfactant properties of this type of compounds are generally desirable for disinfectant and cleaning formulations[108,220–222], but there are also examples of its use in pharmaceutical formulations as well as in cosmetics[109,223–225]. Hence, considering the potential applications of this type of compounds, its use in the disinfection of surfaces was evaluated.

The European standard methodology provided by the OECD "Guidelines for the Testing of Chemicals" - "Quantitative Method for Evaluating Bactericidal Efficacy of Biocides Used on Hard Surfaces"[226], was adapted in order to test the effectiveness of compound **1** in the disinfection of various surfaces: PVC, low density-fiberboard (LDF),

metal (aluminum) and fabric (pure cotton and cotton 65% / polyester 35%). Several variations to the determination of surface sterility after treatment were attempted, and the most reproducible results were obtained by performing a swab sampling of the surface and placing it in growth media. Likewise, diverse methodologies of surface disinfection were tested, apart from the standard methodology. Spray application of the compound solution was tested, since it would be an optimization to its practical application, and the results did not differ from the standard methodology.

The most robust results are also based on observation of bacterial growth at 48 hours, once at 24 hours the reproducibility required for validation could not be obtained. This signify a great reduction of the bacterial load in the tested surfaces, but its complete disinfection could not be achieved. These results compromise the effectiveness of the application of the compound to surfaces contaminated with *B. cereus* ATCC 14579.

The methodologies applied are described in detail in Methodologies, and the results obtained are summarized in the following table:

Table 10. Study on the efficiency of compound **1** in the disinfection of surfaces contaminated with *B. cereus* ATCC 14579

Material	**PVC**	**LDF**	**Metal (aluminium)**	**Fabric (cotton)**	**Fabric (cotton : polyester)**
Method 1	ir	>256	ir	>256	>256
Method 2	>256	nd	nd	>256	>256
Method 3	>256	>256	>256	>256	>256
Method 4	1000	1000	ir	>1000	>1000
Method 5	1000	nd	nd	1000	1000

*MBC values here presented (μg/mL) are the result of observation at 48h. Results were not determined (nd) due to methodology limitations and inconclusive results (ir) due to lack of reproducibility. All experiments were performed with a total of 5 replicates.

The fact that it was not possible to achieve the complete sterilization of surfaces at the expected concentrations may be related to the spore form of bacterial cells produced under harsh conditions[217,227]. It is plausible to assume that bacterial cells devoid of any growth medium when on a surface, begin to produce endospores. The fact that a significant reduction in bacterial load was achieved also supports this hypothesis because only a small amount of the bacterial population would have time to produce endospores.

Also, it would be in accordance to the fact that the most reliable results would be achieved at 48h, due to the lag phase of spore germination and growth to visible colony.

5.2.3 Efficacy against bacterial spores

Bacillus genus is reported as a spore-producing strain, in face of the previous results and in order to evaluate the validity of our hypothesis, the susceptibility of *B. cereus* spores to compound **1** was studied.

The production of spores from strain model was carried out by incubation at 37 ºC in sporulation medium (CCY) for 48 hours[228]. After the purification of the spores by centrifugation, the susceptibility assays to compound **1** were performed. Comparing the susceptibility observed for vegetative cells and for spores, over diverse stages of the germination procedure, the results presented in table 11 were obtained.

Table 11. Susceptibility to compound 1 of diverse cellular forms of *B. cereus* ATCC 14579

***B. cereus* ATCC 14579**	**MBC (µg/mL)**
Vegetative cells	32
Spores	> 256
Vegetative cells suspended in 1 mM PBS and 0.01 % tween 20	16
Spores suspended in 1 mM PBS and 0.01 % tween 20	>256
Vegetative cells after heat treatment at 70ºC for 30 minutes	8
Spores after heat treatment at 70ºC for 30 minutes	16
Vegetative cells in AGFK buffer* at 37ºC for 20 min	8
Spores after germination, achieved in AGFK buffer* at 37ºC for 20 min	16

*AGFK buffer – germination inducer buffer (30 mM L-asparagine, 5,6 mM D-Glucose, 5,6 mM D-Fructose, 20 mM KCl and 50 mM Tris-HCl, pH 8,4)

The results show that compound **1** is bioactive only in vegetative cells, but it is important to note that the spore solution after thermal activation and germination becomes susceptible to the action of the compound. These results could justify the difficulties in surface sterilization, once there's indication that the spore coat limits the access to any important structural component of vegetative cells, and only as soon as they become available, the bactericidal action occurs. Also, this is indicative that access to the cytoplasmic membrane or its constituents is imperative for antibacterial activity.

6 The study of the mode of action

No literature was found with a suitable methodology stating the intention of studying the mechanism of action for this type of compounds. Most of the studies approached these compounds strictly as surfactants, where only the surfactant characteristics were addressed. Furthermore, these studies assumed the observed activity to be solely due to their surfactant capacities and not much discussion was found on the subject.[142,145,146,229] Some of the presented studies discussed a mechanism of action, but analysis was based on their capacity to de-organize the membrane, and this would be causing the cellular lysis observed[230].

The interaction between alkyl glycosides and biological membranes is largely known, and such compounds can be used, for example, as protein solubilizing agents[231,232]. Effects in biological membranes are to be expected for all surfactant compounds, affecting the organization and thermotropic properties of lipids but not all disruptive effects are associated to a negative biological impact: alkyl glycosides can act as permeability enhancers on human cell lines allowing improved cell recovery[233].

Since this new type of alkyl glycosides is associated with a biological activity profile that differs from the traditional, as described before, in order to study its mode of action, a logic course of methodologies was decided upon, exploring the possibility of a non-surfactant effect to be present. Some limitations to the experimental design were imposed by a lack of labelling capability for these compounds. Early experiments with compound **G9** (Table 7) where the aliphatic chain was substituted in the terminal position, a complete loss of bioactivity was observed, a result in agreement with the literature[211].

The first approach taken was aimed at better understand the type of antimicrobial activity, as well as characterize it, employing a time-kill assay. At the same time, with this study we attempted to establish a suitable methodology for comparing the bioactivity of potential candidates to lead compounds, where standard susceptibility assays do not provide the necessary sensibility.

Finally, a multiphasic approach was used in an attempt to unravel the mechanism of action for these compounds, while trying to go beyond the traditional assumption that the biological activity is only due to the surfactant capacity, since the observed microbial specificity at specie-level does not correlate to the bibliography data on other alkyl glycosides, as discussed before. With the consideration that a species-selective biological

activity could become important for drug development in the future[40], understanding the mechanisms behind the bioactivity profile is important.

6.1 Establishing the mechanism of action

The first objective of this study was to verify the type of the antibacterial activity observed and determine a set of parameters to allow the selection of the best lead compound, in the presence of several structures leading to the same MIC and MBC profiles. To achieve this, a time-kill assay methodology was decided upon, since the information on the kinetic parameters obtained through this type of assay could be a strategy to differentiate very similar compounds.

6.1.1 Bactericidal or bacteriostatic action?

In order to evaluate the antimicrobial effectiveness of any lead compounds, a time-kill assay was performed. Cell concentrations (10^6, 10^7 and 10^8 CFU / mL) of the reference strain *Bacillus cereus* ATCC 14579 were exposed to a range of concentration of the desired compounds for determination of time exposure on the number of colony forming units (CFU). The tests were made for each concentration at 35 °C in microplate (liquid medium) in 2 biological and 6 technical replicates. Tests were performed with a temporal resolution of 10 min during the initial 6h of incubation followed by an endpoint reading after 11h of incubation.

This method was firstly applied to compound **1**, the lead chosen from past studies as referred before. For this reason and for being readily available, it was the compound selected for all the mechanist studies, allowing a better integration of results, considered to be a representative of this new family of compounds. The most relevant measurement of antibacterial action kinetics is the T99.9, which is the time necessary for a certain compound to achieve a 99,9% reduction in the bacterial population, a measurement of the cellular impact of a compound widely used in research[234].

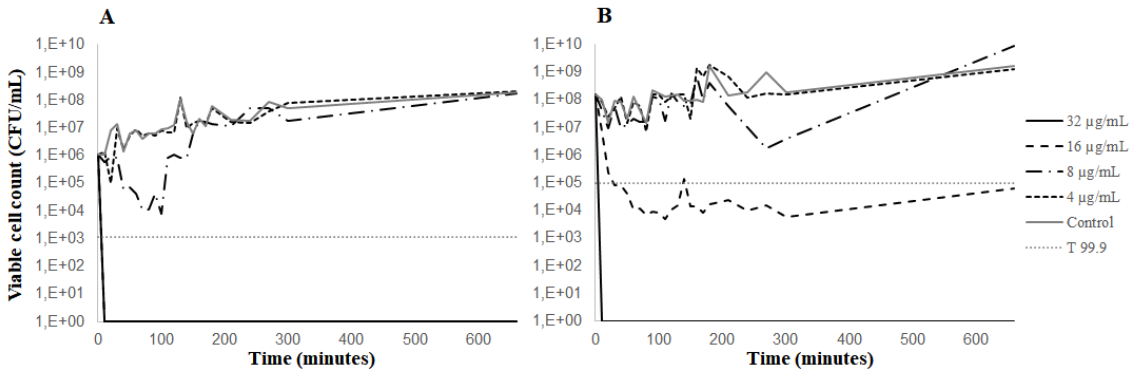


Figure 9. Graphical representation of the variation in B. cereus *ATCC 14579 bacterial vitality with time of exposure to the action of compound 1 (starting inoculum: A – 106 CFU/mL; B - 108 CFU/mL)*

Compound **1** showed a very fast kinetic of bacterial death for the concentration above the MIC, [32 µg/mL], achieving T99.9<10 min. (the smallest time interval allowed by the experimental procedure) (Figure 9). This denotes a highly efficient bactericidal action for this compound, since no viable CFUs could be detected after this very short time of exposure, while if it was a bacteriostatic effect number of CFUs would remain the same after exposure.

The efficacy of this bacterial action is remarkable, once even testing a 100X higher concentration of initial cells than standard conditions resulted in a T99.9 value of < 40 minutes.

6.1.2 Comparison of bactericidal efficiency

The time-kill assay allowed to establish the kinetic parameters of bactericidal activity, so, a comparison assay was performed between compound **1** and a second compound with a similar nature, that exhibited a lower toxicity profile (compound **18**), which made this compound a suitable lead candidate. The tables below contain the bacterial death kinetic data obtained for both compounds **1** and **18**. As an example of the bacterial time kill curves obtained, figure 10 shows the time kill curves for the standard initial bacterial concentration (10^6 CFU / mL).

Table 12. Study on the bacterial death kinetics for *B. cereus* ATCC 14579 in the presence of compounds **1** and **18** (16 µg/mL)

[CFU/ml]ini	Compound	T99.9 (min)	ΔLog (CFU/h)	ΔLog (CFU/24h)	MBC (µg/mL)
10^6	1	<10	6	6	16
	18	>24h	0.5	0	32
10^7	1	180	2	7	16
	18	>24h	0.5	0	32
10^8	1	40	4	4	32
	18	>24h	0	0	32

Table 13. Study on the bacterial death kinetics for *B. cereus* ATCC 14579 in the presence of compounds **1** and **18** (32 µg/mL)

[CFU/ml]ini	Compound	T99.9 (min)	ΔLog (CFU/h)	ΔLog (CFU/24h)	MBC (µg/mL)
10^6	1	<10	6	6	16
	18	90	1	6	32
10^7	1	<10	7	7	16
	18	90	2	7	32
10^8	1	10	8	8	32
	18	90	1	8	32

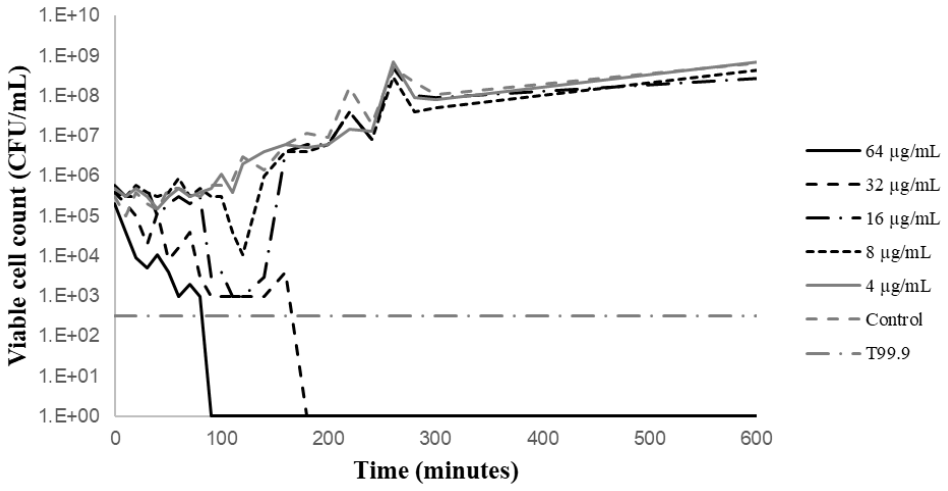


Figure 10. Graphical representation of the variation in B. cereus *ATCC 14579 bacterial vitality with time of exposure to the action of compound 18.*

Compound **1** had been showed to be more effective bactericidal than compound **18**, presenting lower T99.9 values, either at MIC or supra-MIC concentrations. Regardless, both compounds demonstrate the same break-point behavior in the time-kill assays, meaning that at the concentrations that the compound is active, we can observe a complete cellular death, otherwise the presence of the compound does not have an effect in bacterial growth. This could be indicative of similar mechanisms of action.

6.1.3 Bacterial viability

In order to verify and further characterize the bactericidal nature of the bioactivity displayed by compound **1**, a viability assay was devised using flow cytometry, where a single-cell resolution was achieved by hydrodynamic focusing. Cellular viability was determined by using the DNA intercalating agent SYTOX green[235], which can only be incorporated in the bacterial cells once the membrane integrity is compromised. In this way there is a direct correlation between the fluorescence intensity and cellular leakage, as a marker of cellular viability, or the lack thereof. The results obtained are shown in figure 11 and table 14.

B. cereus ATCC-14579 | *B. cereus* ATCC-14579 with compound **1**

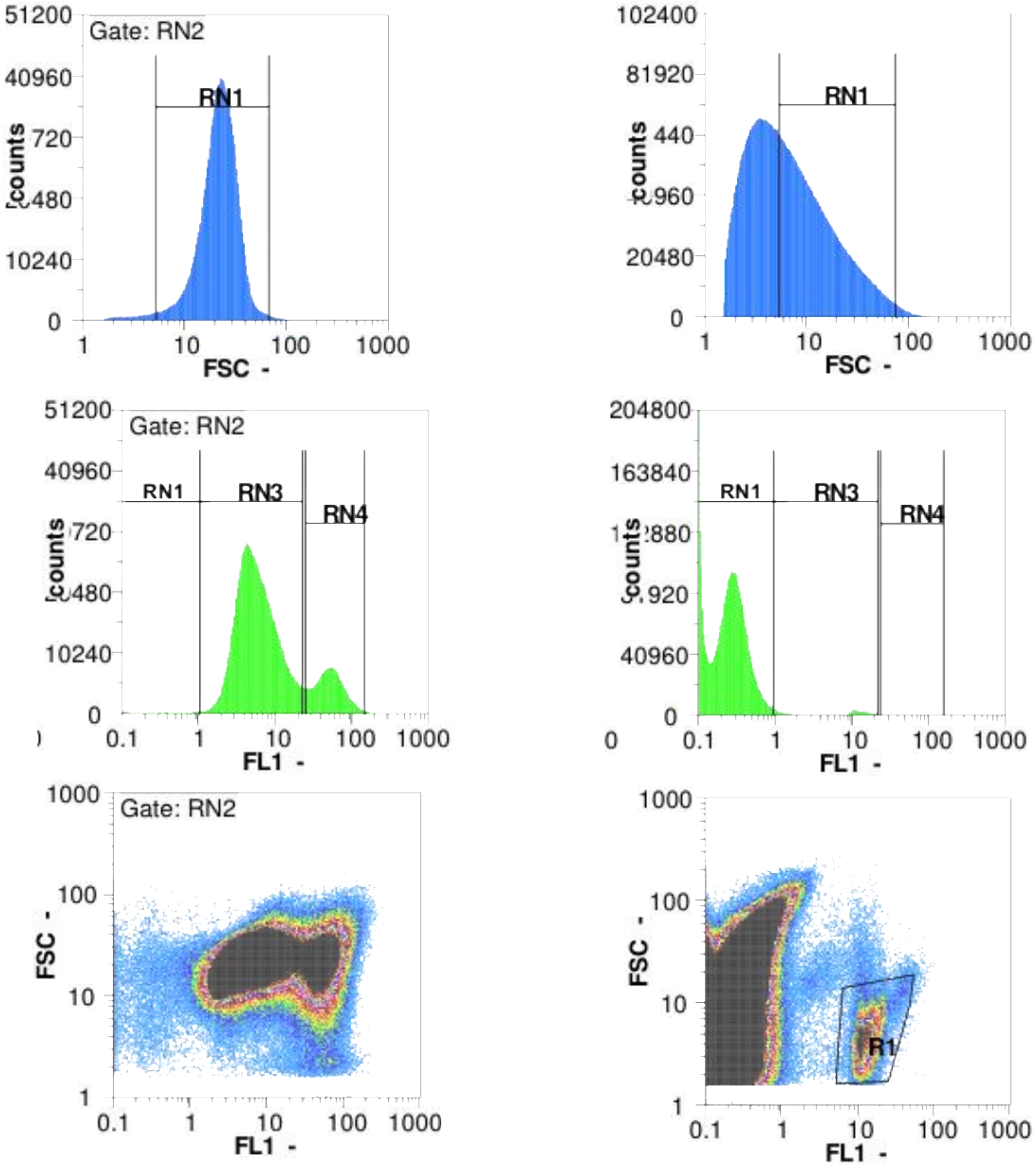


*Figure 11. Impact of compound **1** (256 mg/L) in the cell morphology and viability of* B.cereus *ATCC-14579. FSC - Forward Scatter Channel. FL1 – Fluorencence Level Channel 1. Logaritmic scale (calibration: 1um = 10 FSC Units; 3um = 30 FSC Units). Calibration performed with solution of SYTOX in growth media;*

Table 14. Parametric evaluation by flux cytometry of compound 1 impact in the physiology (FSC) and cellular viability (FL1-SYTOX) of *B. cereus* ATCC-14579

Sample	**FSC geometric mean (FSC; CV)**	**RN3 Viable subpopulation (GM; CV)**	**RN4 Non-viable subpopulation (GM; CV)**	**RN1 sub-cellular events (GM; CV)**	**Number of events analyzed**
B. cereus ATCC 14579	21.42; 41.2%	80.5% (5.76; 68.05%)	16.47% (4.35; 45.69%)	-	$1.6X10^6$
B. cereus ATCC 14579 + compound 1	6.86; 130.34%	1.87% (5.59; 71.5%)	0.12% (38.11; 36.37%)	98.01% (0.26; 52.72%)	$5.5X10^6$

FSC: Foward Scatter Channel; CV = Covariance; GM = Geometric Mean

The analysis of cellular viability comes from SYTOX fluorescence (channel FL1 – 536/40nm) allowing the identification of the sub-populations considered as viable cells, shown in gate **RN3** (1 – 22 FL1 Units), and as non-viable cells, show in gate **RN4** (25 – 160 FL1 Units).

Firstly, the average size of the particles analyzed in the exponential growth *B. cereus* sample, without the presence of compound, was of 2.1 µm (geometric average: 21.42 FSC units CV = 41.2%), an expected size for this bacterial specie, while in the presence of compound there is a non-normal distribution of particles with much lower size, probably resulting from cellular debris, causing the low-intensity fluorescence observed in FL1-RN1, as well as possibly micellar or vesicular ultrastructures formed by compound 1.

The analysis of the particle size (FSC) versus cellular viability (FL1 -SYTOX) has revealed the almost total absence of cells when submitted to the compounds action, leaving only a small refractory population (R1 (1,87%), that could be constituted by death cells that retained their features or cells in lysis process. This data supports the microscopic observations, confirming the bactericidal effect and consequent complete bacterial cell lysis, not allowing any further visualization.

6.1.4 Bacterial vitality of a refractory population

6.1.4.1 Defining cellular vitality

Although the definition of cellular viability is generally well defined as the ability of a cell to grow and multiply[236], the definition of cellular vitality less well defined. Nonetheless, vitality is regularly associated to cellular fitness or metabolic activity[236,237].

So, for the purposes of this work, cellular viability will be considered de capacity of a cell to grow and multiply and can be determined by assays that assess cellular integrity, since if this characteristic is lost it is impossible for these cells to regain viability. Vitality on the other and will be considered as an indicator of cellular metabolic activity, and so, determined by metabolic markers.

6.1.4.2 How alive is a refractory population?

The observation of a refractory cellular population by flux cytometry, after the action of compound **1**, raised the question if it consisted of dead bacterial cells that simply retained their bacterial structure or if it is indeed a population of cells putatively resistant to compound **1**. Hence, a better understand of the impact of this compound in bacterial vitality at the single cell level, in real time and independent of their cellular integrity is needed.

With this objective, an *in-situ* real-time fluorescence microscopy approach was employed, where *Bacillus subtillis* 3A40 was used since it expresses GFP (Green Fluorescent Protein) and this expression is controlled thru a promotor inducible by IPTG (Isopropyl β-D-1-thiogalactopyranoside) in a constitutive manner. This way, the protein expression is independent of external factors, constant and an indicator of active metabolic processes and cellular fitness. With this approach, the visualization of GFP fluorescence should provide a reliable and prompt measurement of cellular vitality at single cell level in real-time.

Another determining factor to the success of this approach, in order to obtain the single-cell real-time sensitivity, is cellular immobilization, and to that end, several methodologies were tested, namely: immobilization in agar matrix and chemical immobilization.

Relating to the latter, immobilization with polydopamine[238] and polyethylenimine[239] were tested, but it was observed that both these systems

compromised bacterial integrity or, at least, altered their susceptibility and, for this reason, they could not be used.

Regarding the immobilization in agar matrix, it was used a low melting agar and whether by immobilizing the bacteria in an agar matrix[240,241] and then expose them to compound **1** or by previous integration of the compound in the matrix and further inoculation of the matrix, this alkyl 2-deoxy glycoside has demonstrated some issues in exhibiting its bactericidal action in this environment.

Efforts to optimize such methodologies to the compounds action proved unsuccessful, and so, observation of the compounds' action without the presence of an immobilization device was performed. These results remained diverse from the expected, so, it was postulated that being in a microscope preparation fundamentally changed the compound's bactericidal capacity. Such effects could be due to a necessary "surface:solution" ratio in order to fully express their biological activities. This could be indicative of a necessity of this type of compounds to create ultrastructures and, these are, at least, partially responsible for the bioactivity observed.

Given the inability to observe the *in loco* action of the compound in real time, a bacterial culture was submitted to the action of compound and hourly samples microscopically observed, resulting in a reproducible and consistent methodology. Even if real-time observation could be achieved, the approach taken produced the proposed results. Examples of the images obtained are shown below.

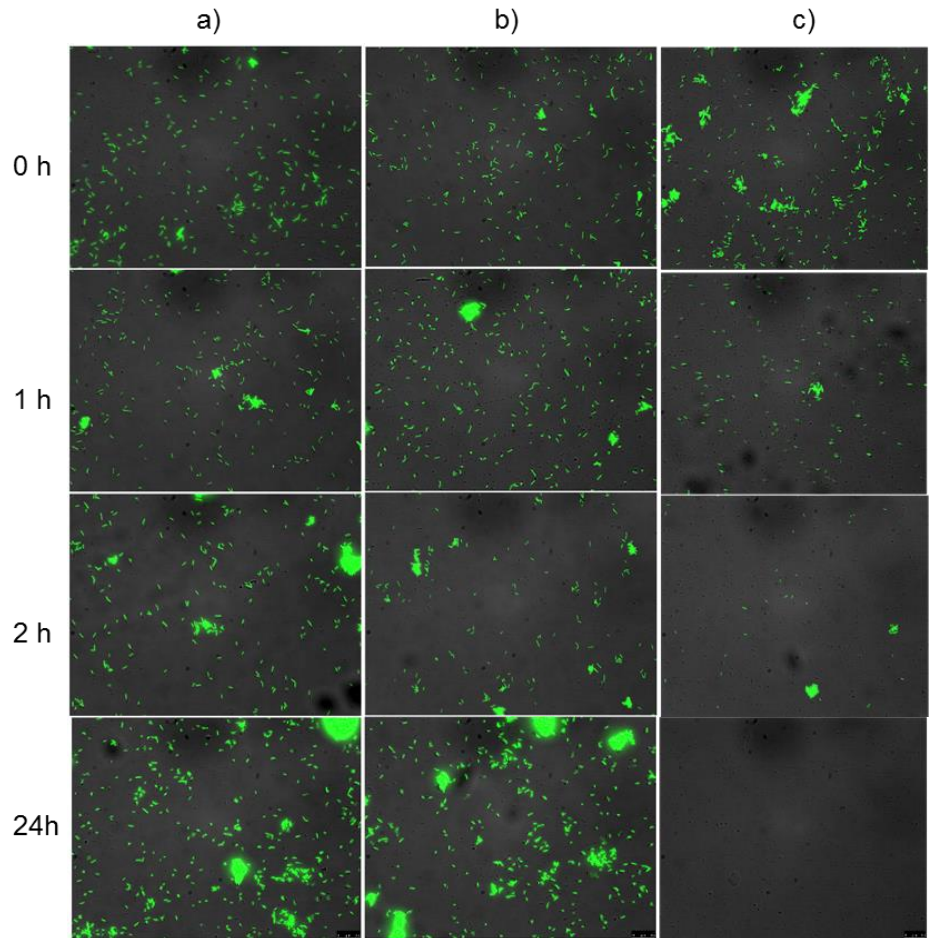


*Figure 12. Images obtained from different temporal points, in 3 experimental conditions: a) bacterial culture b) Bacterial culture with DMSO 6% c) bacterial culture with compound **1** [256 µg/mL]*

These images show a clear loss of cellular vitality over time, with exposure times over 2 hours showing GFP expressing cells only survive in clusters (detailed image in Annex 5). For a better statistical analysis of the data obtained, automated total cell count and GFP expressing cell count was performed and the results are presented in table 15 and in the graphic below (Figure 13).

Table 15. Impact of compound **1** in cellular vitality, observed by GFP fluorescence.

Time (h)	Control			Compound 1		
	Total cells*	Metabolically active cells**	Vitality rate	Total cells	Metabolically active cells	Vitality rate
0	196 ±43,8	189 ± 39,3	96,4%	397 ±139,7	372 ±124,2	93,7%
1	292 ±53,9	277 ±53,6	94,9%	328 ±65,4	292 ±54,1	89,0%
2	147 ±51,7	127 ±44,4	86,4%	91 ±36,5	60 ±28,3	65,9%
3	387 ±138,3	358 ±113,4	92,5%	140 ±44,9	50 ±15,3	35,7%
4	235 ±46,7	216 ±38,1	91,9%	226 ±100,2	56 ±16,7	24,8%
5	362 ±68,1	304 ±52,8	84,0%	167 ±54,0	13 ±13,9	7,8%
24	250 ±39,0	237 ±31,3	94,8%	97 ±33,2	1 ±1,3	1,0%

*Median of total cell number (n=5) observed using DIC field ± Variance
** Median of the cell number (n=5) expressing GFP (Excitation/emission = 495/517 nm) ± Variance

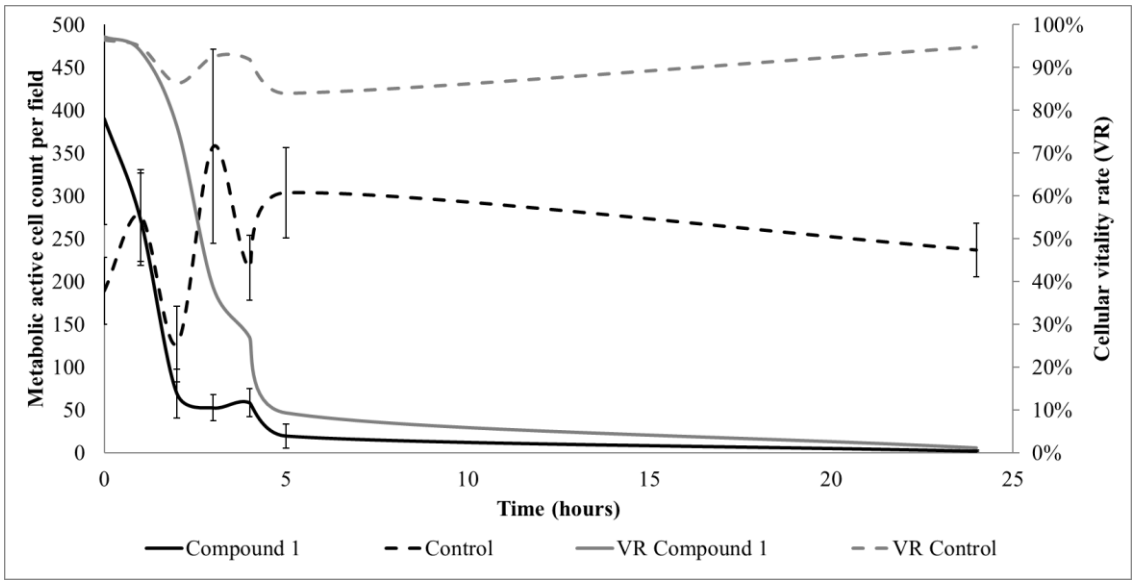


*Figure 13. Impact of compound **1** in cellular metabolic activity with time, as observed by fluorescence microscopy. Compound 1 – bacteria exposed to compound **1**; Control conditions – bacteria exposed to similar concentration of DMSO*

The attempt to observe the bactericidal effect of this type of compounds in real time proved to be unfruitful, but, despite of that, the data obtained confirmed the type of effect compound **1** has on bacterial cells and the visualization of the loss of vitality over time was achieved.

6.1.5 Efficacy against antimicrobial-resistant mutants

When two substances have similar or related modes of action, the emergence of resistance to one of them will lead to a reduction of susceptibility to the other. This is known as cross-resistance, and it allowed the search for an association between the mechanisms of action of the main antibiotic families and the mode of action of compound **1**. Positive or negative variations of susceptibility to this compound would provide important guidelines in the search of the molecular target of its bactericidal action.

Thus, a set of spontaneous mutants from *B. cereus* ATCC 14579 bacterial strain were created by subjecting the parental strain to increasing concentrations of chosen antibiotics for about 15 days or, until a minimum increase in MIC values of 10 times was observed, using a daily serial passaging methodology with gradient plates (Table 16).

The same procedure was applied with compound **1**, to assess the capacity for the *B. cereus* strain to acquire resistance by natural mutagenesis. After 15 days of serial passaging in gradient plates supplemented with the compound, no significant resistance was observed, with MIC values remaining the same by the end of the experiment. This result represents a major advantage of alkyl deoxy glycosides, since after the same time of exposure in the same experimental conditions, it was possible to obtain resistant mutants to all the antibiotic classes but not our compound.

The bacterial resistant strains susceptibility to compound **1** was then determined, and the results obtained are presented in the following table:

Table 16. Susceptibility of resistant strains.

		Compound 1	
Antibiotic Resistant Mutant		**MIC (µg/mL)**	**MBC (µg/mL)**
Erythromycin	(20 µg/mL)*	16	16
Penicillin	(20 µg/mL)*	16	16
Vancomycin	(5 µg/mL)*	16	16
Ciprofloxacin	(50 µg/mL)*	16	16
Tetracycline	(50 µg/mL)*	16	16

* () final MIC values of the resistant mutants obtained

The values of MIC and MBC obtained were equal to the values determined for the standard strain showing that there is no correlation between the mechanism of action of these drugs (and the classes they represent) and the mode of action of the compound **1**.

6.1.6 Main metabolic pathways affected by alkyl deoxy glycosides

In order to obtain a number of possible directions regarding possible molecular targets while also considering the possibility of a general surfactant effect, an experiment was designed based in the differential analysis of a phenotypic microarray assay, focused mainly in different carbon sources available to *B. cereus* ATCC 14579 bacteria.

To assess the phenotypic cellular response to the presence of the compound in study, the "Phenotype Biolog® microarray" technology, was adapted to evaluate the impact of compound **1** in the metabolism of 95 different carbon sources. The 95 different carbon sources selected to the assay were the ones designed as PM1 according to the Biolog technology (Annex 6). Four replicates of this plate were used and after the assembly of the used media according to the manufacturer's instructions[242], the media of each plate was supplemented with increasing concentrations of compound **1**, namely 0 μg/mL, 4 μg/mL, 8 μg/mL and finally 16 μg/mL, the MIC value obtained. Thus, this system allowed not only to observe the effect of the compound in the metabolism, but also if the effect occurred in a concentration-dependent manner.

The evaluation of the bacterial metabolic fitness was assessed by NADH reduction via a dye mixture supplied by the manufacturer, that function as tetrazolium dye, meaning, an indirect measure of cellular capacity to oxidise NADH to NAD^+, causing a reduction of the dye resulting in the emergence of coloration in solution[243].

This coloration was measured by OD_{590}, in a microplate reader, hourly, and the results obtained were treated and plotted in order to better visualize the impact of compound **1** (Annex 7).

For each different carbon source, we observe the differential response to the compound **1**, and so, in order to understand what carbon sources were significant to the bioactivity, analysis of the data obtained was performed as described in the methodology section, and the results were classified according to 5 categories: 1) highly inhibitory effect (above 5σ diminution in OD values), 2) inhibitory effect (under 5σ but above 2σ diminution in OD values), 3) no effect (under 2σ variation from the OD values of the control condition), 4) growth potentiation effect (with a rise of more than 2σ in OD values), and finally 5) no growth condition, when the OD values for the control condition and all the concentrations were similar to the no inoculation condition. It was this no inoculation condition, where the OD variations resulted solely from abiotic reduction of

the dye, that allowed for the normalization of the values, and the definition of the cut-off σ values, since it provided a cross-experiment stable control and so using these standard variation values (2σ and 5σ) would provide suitable confidence intervals.

After this classification, the carbon sources associated to inhibitory effects 1) and 2) were associated with the respective metabolic models and information processing systems, known for the model strain used (*B. cereus* ATCC 14579) and available in the database "Kyoto Encyclopedia of Genes and Genomes" (KEGG). A simple rating system was used, where the metabolic pathways were ordered according to the number of associated carbon sources that produced an inhibitory response.

Using this database, we determined the metabolic pathways and other systems with a measured response associated with the action of compound **1** (Table 17), revealing as the most promising molecular target candidates the environmental information processing systems (ABC and phosphotransferase transport systems) and the metabolic pathways of starch and sucrose.

Table 17. Main cellular systems associated to the results obtained by deferential phenotypic microarray data analysis

Metabolic pathway	Number of compounds associated
ABC transporters	13
Starch and sucrose metabolism	7
Phosphotransferase system (PTS)	7
Aminoacyl-tRNA biosynthesis	6
Methane metabolism	5
Two-component system	5
Pyruvate metabolism	4

These results provided a general and integrated idea of what cellular targets could be disrupted and form a theory for the mechanism of action, and since most of the systems referred are an initial step in the intake of nutrients and information from the environment, a disruption of these systems could lead to impairment of all the metabolic pathways dependent on them, leading to cellular death.

Also, for each different carbon source, we observed a differential response to the compound **1**: for the same concentrations of compound, depending on the carbon source,

we can observe total inhibition of bacterial growth, no effect at all or even potentiation of growth at subinhibitory concentrations of this type of compounds. Such responses have provided more meaning to the search of a molecular target, since if a simple surfactant effect was present, changes to the potency of this unspecific effect could be expected but not the complete inactivity of the compound. This diversification of the results also suggests that this compound acts on a main cellular system that may or may not be activated depending on the environment.

In order to understand the hierarchical relationship between the aforementioned pathways as well as the existence of cellular, molecular or metabolic global connections, the data obtained was treated and a reconstruction model of the molecular pathways (metabolic and others), designed based on the determination of non-parametric association between the consumption of the various carbon sources previously analyzed. Metabolic reconstruction was performed through the application of graph theory with hierarchical superimposition of nonparametric coefficients determined previously. With this methodology, an extensive map was obtained (Annex 8) that represents the association between the impact of compound **1** and several molecular pathways, as well as, associations between them and other cellular structures.

It was observed the convergence of the hierarchical routes obtained to a main node classified as ABC transporters systems, also surrounded by other secondary hubs (PTS systems and amino acyl t-RNA biosynthesis for example), but that are in accordance to the data presented before. Cellular transporters as well as most of the other possible targets are described by the used model and by literature as present in the bacterial cytoplasmic membrane, suggesting that compound **1** primary targets are one or more constituents of the cell membrane. Inactivation of these primary targets may lead to malfunction or inactivation of various metabolic pathways, resulting in the cascade of effects observed and ultimately in cell death. However, we must also consider that the observed effects could be the result from a non-specific interaction with the cytoplasmic membrane, which results in a similar cascade reaction, even if it does not explain all the characteristics observed for this alkyl glycoside bactericidal action.

6.1.7 Investigating the mode of action by genetic dissection

Genetic studies are an essential tool in the discovery of a mechanism of action and allow the definitive confirmation of any specific molecular target. The development of mutants resistant to the molecules in study is one of the strongest proofs that their biological activity is dependent of the modified molecular targets[244]. Once the modified genes are determined, which translates to a modification in the potential molecular target, several new studies can be performed. The overexpression or modification of that target allows the complete understanding of the interaction of the small molecule with the target, thus permitting the rational design and optimization of said small molecules.

With this in mind, a genetic approach was decided upon. Since the results from the differential metabolomic study provided various possible targets and reconstruction model indicated a number of different hubs affected by the presence of compound **1**, a mutant library by random transposition was created, introducing multiple modifications across the entire genome, as an attempt to discover possible genetic modifications that can lead to the emergence of resistance or diminishing susceptibility to the small molecules in study.

Also, since the cellular transport systems (ABC and PTS) were the main targets obtained from the approaches described earlier, a specific knockout mutant library with selective inactivation of these various transporter system proteins was obtained, in order to clearly establish the relevance of these systems to the bactericidal action observed.

For the creation of a mutant library by random transposition, we used the reference strain *B. cereus* ATCC 14579 and the plasmid pLVT1, carrying the transposon Tn917. Upon electroporation and mutant selection, 2 mutant libraries were obtained with a significant number of mutants, of about $9.8x10^8$ total mutant cells, amounting to 10^6 mutants per ORF and representing a transposon mutagenesis yield of 98%.

Regarding the specific knockout mutant library, firstly, a study on the conserved genome and proteome of the selected *Bacillus* species was carried out, aiming to identify which proteins were associated to the transport systems that were the most relevant targets from the metabolomic study. From these proteins, a list of specific mutants was selected, both from ABC transporter and PTS systems, and the knockout mutant library was obtained, with the parent strain *Bacillus subtilis* 168. The susceptibility of all the mutant libraries was evaluated, and the results are presented in the table 18.

Table 18. Obtained mutants and susceptibility to compound **1**.

Strain / Library	Description	MIC (μg/mL)	MBC (μg/mL)
***Bacillus cereus* ATCC 14579**	Parental isogenic strain	16	32
Mutant Library 1	Mutant library generated by random transposition using pLTV1 system	16	16
Mutant Library 2	Mutant library generated by random transposition using pLTV1 system	8	8
***Bacillus subtilis* 168**	Parental isogenic strain	16	32
***Bacillus subtilis* ΔygaD**	Putative ABC transporter (ATP-binding protein)	16	16
***Bacillus subtilis* ΔyjdD**	PTS mannose-specific enzyme IIBCA component	16	16
***Bacillus subtilis* ΔyhcJ**	Putative ABC transporter (binding lipoprotein)	16	16
***Bacillus subtilis* ΔyhaQ**	Na+-efflux ABC transporter (ATP-bindingprotein)	16	16
***Bacillus subtilis* ΔykfD**	Putative cell wall oligopeptide ABC transporter(ATP binding protein)	16	16
***Bacillus subtilis* ΔykoD**	Thiamine-related ABC transport system ATP-binding protein, thi-box regilon	16	16
***Bacillus subtilis* ΔywhQ**	ABC transport system ATP-binding protein, subtilosin production	16	32
***Bacillus subtilis* ΔyknU**	Putative ABC transporter (ATP-binding protein)	16	32
***Bacillus subtilis* ΔykpA**	Fructose-1-phosphate kinase(similar to ABC transport system ATP-binding protein)	16	32
***Bacillus subtilis* ΔyheH**	Multidrug ABC transporter (ATP-binding protein), involved in the signalling pathway that activates KinA during sporulation initiation	16	32
***Bacillus subtilis* ΔyvcC**	Multidrug ABC transporter	16	32
***Bacillus subtilis* ΔywbA**	Putative phosphotransferase system enzyme IIC permease component	16	32
***Bacillus subtilis* ΔyxdM**	ABC transporter (permease); efflux of cationicpeptides	16	32
***Bacillus subtilis* ΔyybL**	Putative integral inner membrane protein	16	32

***Bacillus subtilis* ΔybbF**	Putative sugar phosphotransferase enzyme II	16	16
***Bacillus subtilis* ΔmtlA**	Phosphotransferase system (PTS) mannitol-specific enzyme IICB component	16	32
***Bacillus subtilis* ΔexpZ**	ABC transport system ATP-binding protein, export of virginiamycin M and lincomycin	16	32
***Bacillus subtilis* ΔydhO**	PTS oligo-beta-mannoside-specific enzyme IIC component	16	32
***Bacillus subtilis* ΔyfmR**	Putative ABC protein involved in RecA-independent precise excision of transposons	16	16
***Bacillus subtilis* ΔtreP**	Phosphotransferase system (PTS) trehalose-specific enzyme IIBC component	16	16
***Bacillus subtilis* ΔyfiB**	ABC transporter ATP-binding protein	32	32
***Bacillus subtilis* ΔyfiM**	Putative ABC transporter (permease)	16	16
***Bacillus subtilis* ΔyflF**	PTS N-acetylglucosamine-specific enzyme IICB component	16	32
***Bacillus subtilis* ΔyfiC**	Putative ABC transporter (ATP-binding protein)	16	16
***Bacillus subtilis* ΔyfiN**	putative ABC transporter (permease)	16	32
***Bacillus subtilis* ΔybdB**	Exporter of cell killing factor, ABC transport system permease protein, the mutation exhibited highly oligosporogenous phenotypes	16	32
***Bacillus subtilis* ΔybdA**	Exporter of cell killing factor, ABC transport system ATP-binding protein, the mutation exhibited highly oligosporogenous phenotypes	16	32

The MIC and MBC values obtained lack of biologically significant difference (a difference in values $>\log_2$) when compared to the parent strains, or the general biological activity observed for *Bacillus* species. These results are indicative of the absence of a specific molecular target for the mechanism of action, or at least one that is not essential, since these methods cannot evaluate essential targets, once mutations in them do not produce viable mutants.

6.1.7.1 The impact of ATP-dependent systems

The genetic study previously described showed no correlation between the bioactivity of compound **1** and any non-essential molecular targets, including from the main clusters resulting from the metabolic correlation map obtained previously. Nonetheless, several other metabolic pathways were indicated as putative targets, as stated in section 6.1.5, as well the fact that only non-essential targets could be evaluated by the genetic methodology applied, and so a complementary approach was devised.

By employing a proton motive force (PMF) inhibitor, an agent that causes the decoupling of proton gradient, the inactivation of all membrane ATP-dependent systems is caused. In order to achieve this, CCCP (carbonyl cyanide m-chlorophenyl hydrazone) was used, since it is well known as a PMF inhibitor as well as capable of impairing the ATP production in the cell, by inhibiting oxidative phosphorylation[238,245]. This way all cellular transport systems were inactivated, and their effect in the action com compound **1** could be determined.

So, firstly, the susceptibility of the strain *B. cereus* ATCC 14579 to CCCP was evaluated, and then, several mixtures with compound **1** were used, aiming to observe a synergistic or antagonistic effect. MIC values for CCCP were determined at 0,625 µg/mL, hence ½ MIC was used in the synergistic study (0,3 µg/mL).

Results show that the MIC values obtained for the control condition (only compound **1** with a MIC of 32 µg/mL) were unaltered by be the presence of CCCP, showing that compound **1** effect was neither positively nor negatively affected by the presence of CCCP. Since no correlation was found between both compounds, this is a strong indication that none of the transport systems, or any other ATP dependent system, interfere with the antibacterial activity observed.

Putting together all the results just presented, we concluded that in fact the transport systems were unlikely to be the target of the action of the compounds here presented, even though they represented the most promising target according to the metabolic assay. Not only that but, since the genetic dissection was the strongest tool for the determination of a possible target, or at least provide a clue to what cellular systems were associated to the compounds' action, the results obtained are a strong indication that the mechanism of action of such compounds is devoid of a molecular target.

Thus, the most prominent hypothesis for the mechanism of action focuses on a physicochemical interaction with the cytoplasmic membrane, destabilizing it and leading to cell lysis, even though a suitable explanation to the selectivity observed cannot be achieved within this traditional point of view.

6.1.8 Bacterial envelope and bactericidal activity

The information obtained by the metabolic reconstruction and the aforementioned genetic studies suggest that most of the results obtained in the phenotypic analysis are associated to a single structural component: the bacterial membrane. For this reason, a study was devised in order to better understand if the bioactivity observed for compound **1**, and maybe more importantly the resistance to it, is influenced by other cellular structures or is only associated to the bacterial membrane.

To this end, the evaluation of the importance of certain bacterial ultra-structures, including wall and cell membrane, was performed recurring to protoplasts and spheroplasts and determining their susceptibility to compound **1**. By using this methodology, it is possible to deconstruct the bacteria, at least its most exterior layers, and assess the impact of said structures in the bioactivity.

Protoplasts and spheroplasts were produced using lysozyme to digest the cell wall of Gram positive and Gram-negative bacteria, exposing the respective cytoplasmic membrane. This way, if any hypothetical targets were present in the outermost layers, higher MIC values should be expected. According to the previous results this should not be expected, instead, since the access to the bacterial membrane is immediate, a reduction of MIC values to the susceptible species is expected.

The bacterial species selected to this assay were two Gram-positive bacteria (the susceptible control strain *B. cereus* ATCC 14579 and the resistant *S. aureus* ATCC 29213) and one Gram-negative (resistant strain *E. coli* K12). With these selected bacterial species, we intended to gain any insight into the mechanisms of resistance of the unexpectingly resistant Gram-positive *S. aureus*, and what causes the general Gram-negative resistance, of which *E. coli* is a representative species (Table 19).

The results of the test carried out are shown in the following table and figure:

Table 19. Activity of compound **1** on *E.coli*, *B. cereus*, *S. aureus*, and corresponding spheroplasts and protoplasts, expressed in MIC, compared with the activity of a control drug (polymyxin B).

Strain	MIC (µg/mL)	Polymyxin B
E. coli K12	256	8
E. coli K12 spheroplasts	4	>1
B. cereus ATCC 14579	16	128
B. cereus ATCC 14579 protoplasts	16	16
S. aureus ATCC 29213	256	128
S. aureus ATCC 29213 protoplasts	256	>1

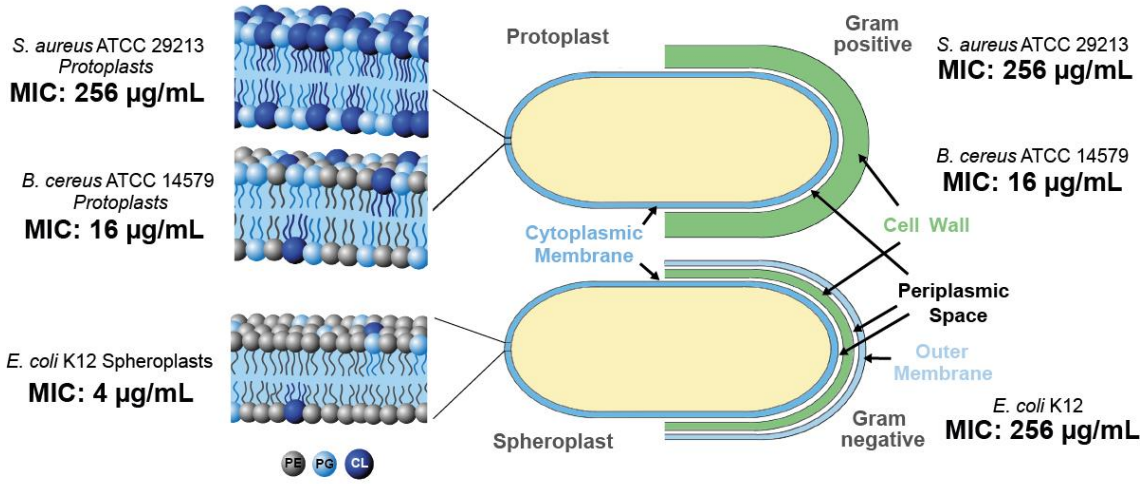


Figure 14. Representation and summary of the results obtained and relation with the bacterial ultrastructures. PE-phosphatidylethanolamine; PG-phosphatidylglycerol; CL-cardiolipin

Regarding Gram negative bacteria (*E. coli* K12 in this case), the structure responsible for the resistance to this family of compounds is probably the cell wall or its external membrane, since compound **1** is not active in the vegetative cell but to its spheroplasts has a MIC value of 4 µg/mL. Such observation denotes that, probably, for most Gram-negative bacteria the main mechanism of resistance is the inaccessibility of the inner membrane to this type of compounds. At the same time, this result also reinforces the idea that the bioactivity is due to the interaction with the cytoplasmic membrane.

As for the results obtained with the Gram-positive bacteria, a conclusion that holds true to both the susceptible and the resistant species is that the external cell wall does not

influence the bioactivity of this compound since the MIC values for were the same for the vegetative cells and their respective protoplasts. This same observation regarding the *S. aureus* strain, shows that the resistance mechanism in this case is not the inaccessibility to the bacterial membrane, but possibly some of its constituents or even its composition that somehow inhibit the supposed surfactant action of these compounds.

This information only adds to the strangeness of the selectivity that is present for these alkyl deoxy glycosides, even though a molecular target for its mechanism of action was not found and the predominant theory relies in a non-specific effect, probably associated to the bacterial membrane. One important hypothesis to take into consideration is the possibility of the specificity of bactericidal action being correlated to the membrane lipid composition (Figure 14). Although similar specificity characteristics are known, as mentioned in section 1.3.1.1 for daptomycin, further studies most be performed in order to confirm this.

6.1.9 Morphologic changes of the cellular envelope by the action of compound 1

Atomic Force Microscopy (AFM) is a suitable technique for precise three-dimensional mapping of microbial cell surfaces and the detection of very subtle changes in their surface morphology and structure[246]. This highly sensitive technique has been demonstrated to be a proficient tool for the assessment of the mechanism of action of bactericides[247,248]. Employing tapping mode provides nanometer-scale resolution in topographic imaging, while also allowing for phase imaging, that can be used to map compositional variations in heterogenous samples. As phase imaging is impacted by factors like viscoelasticity, elasticity and adhesion, it allows to observe the alteration of bacterial surface by action of membrane active compounds.

Hence, such methodologies were employed as means to evaluate and observe, possibly in real time, the impact of compound **1** in the bacterial surface and its morphology, in order to confirm its impact in the bacterial membrane and bacterial cell damaging capabilities.

In order to visualize the desired bacterial surfaces, two methodologies were tested, designated "in situ" and "ex situ"[249]. The technique "in situ" allows to visualize in real

time the cell envelope and any morphological changes caused by the presence of the compound, once it is performed in liquid media.

This would be the best candidate for an assessment of the compounds action, since the observation in real-time would provide an unequivocal proof of the bactericidal effect. However, it was not possible to obtain consistent data once compound **1** was added, and the experiment results varied independent from all the factors verified.

One of the main issues in "in situ" analysis is the need for a fixating agent[250], in order to immobilize the bacteria. The fixating agents tested did not provide reproducible results, possibly by modifying the bacterial susceptibility or compromising the viability and/or vitality as well as possible interactions with the compound or its ultrastructures.

Due to this, an "ex situ" approach provided the best results allowing a good comparison between the observation of bacteria exposed to the action of the compound **1** and a control condition, of healthy cell culture. In this manner, a characterization of the morphological differences caused by the presence of this compound was possible, as shown in figure 15.

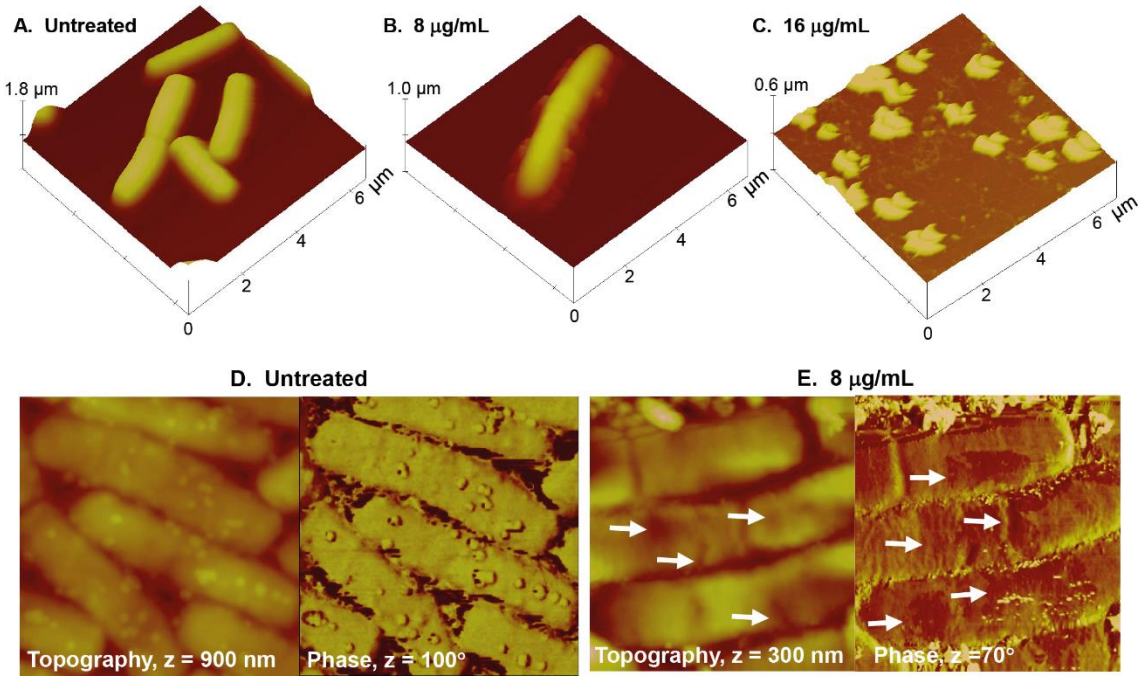


*Figure 15. Observation by AFM of bacterial morphology after exposure to compound **1** at a sub inhibitory concentration [8 µg/mL] (B and E) and comparison to control condition (A and D). Cellular lysis observed at MIC values [16 µg/mL].*

Bacterial suspensions were subjected to the action of the compound for 2 hours, then analyzed by AFM Tapping™ mode. Compound 1 was tested at sub-inhibitory, at MIC value and super inhibitory concentrations. It was observed complete cell lysis at MIC concentrations and higher, without structures that resemble bacterial cells, predominating cell debris (C).

At concentrations below the MIC, there are changes in the morphology of the bacterial surface when compared to the control, however neither the shape nor the morphology of the cell envelope seems to indicate loss of viability. For better observation of the surface, acquisitions with the dimensions 2x2 μm^2 were performed (Figure 15, D and E).

The phase images (right images of D and E), derive from a change in the phase of the vibrating tip. The information in this type of images reflects differences in surface stiffness/softness and adhesion to the tip. In this case, changes in the adhesion due to the release of cellular contents could give rise to the observed contrast at sub-inhibitory concentrations of compound (white arrows). These modifications are evident and most likely correspond to damage of the cell wall in the form of cracks and possible release of cytoplasmic contents.

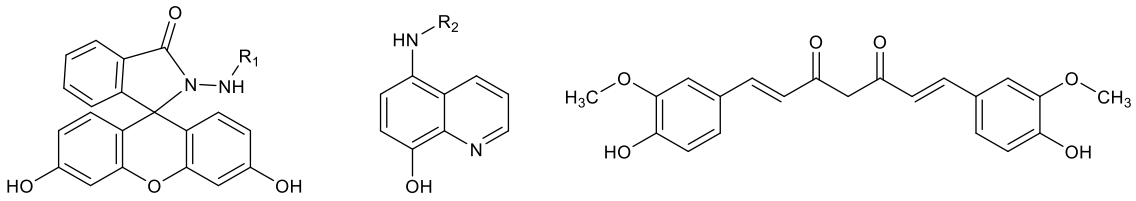
With these observations, it is clear that a bactericidal action is present and that compound **1** causes damage to the cellular envelope, even at leading to cell lysis.

7 Carbohydrate chemistry as an important tool against Alzheimer's disease

Given all the advantages of introducing glucose moieties in bioactive molecules, already discussed before in section 3.2, a glycoconjugation approach was employed to important molecules acting as copper (II) chelators or P-gp inducers.

7.1 Target compounds and synthetic approaches

The primary goal is the synthesis of copper chelating molecules and their glycoconjugates in order to find an alternative for early AD diagnosis via blood testing, a technology patented and under use by Biofordrug, and at the same time develop possible therapeutic alternatives to the treatment and prevention of AD. Three main moieties were selected as potential selective copper chelating molecules with functional groups susceptible to glycoconjugation (Figure 16).



L1 **L2** **L3**

Figure 16. Proposed bioactive sugar acceptors

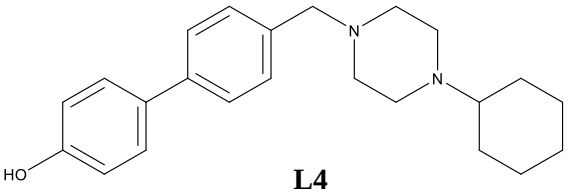
Fluorescein hydrazide derivatives (**L1**), are well known for their copper chelating capacity and application as a fluorescent probes[251,252]. It is also a very interesting moiety regarding its selectivity towards copper, making its glucoconjugate a very attractive molecule with a high potential for therapeutic application.

Compound **L2** is also the scaffold for a number of derivatives developed by Biofordrug with copper chelating properties, but generally with low solubility in aqueous media. Hence, linking a sugar moiety to the chelating molecule is expected to solve this problem, amongst other advantages.

Regarding compound **L3**, curcumin, is a very interesting compound with various known biological activities described, including antimicrobial and anticancer, many of them linked to its capacity to chelate copper and iron. This compound integrates the class of PAINS and has been reported to have the capacity to regulate a number of cellular

processes and modulate transport systems like P-gp. This broad range of biological effects is also a double-edge blade, because several interactions with other drugs are known. Meanwile, any uses are limited by a low bioavailability and solubility in biological media, so, a glucoconjugate derivative could represent very interesting solution to the problem, allowing its use for the desired goals.

The transport system P-gp possibly plays a very important role in AD as discussed before in section 3.1.4, and so it represents an interesting therapeutic target. Even though the real mechanism of transport of P-gp is not completely understood, several compounds have been found to promote the expression of P-gp or increase its activity.[39,40] Compound **L4** (Figure 17) is one of those compounds, found to be a potent promoter of P-gp expression, and capable of penetrating the BBB, a necessary characteristic for Alzheimer's therapeutics.



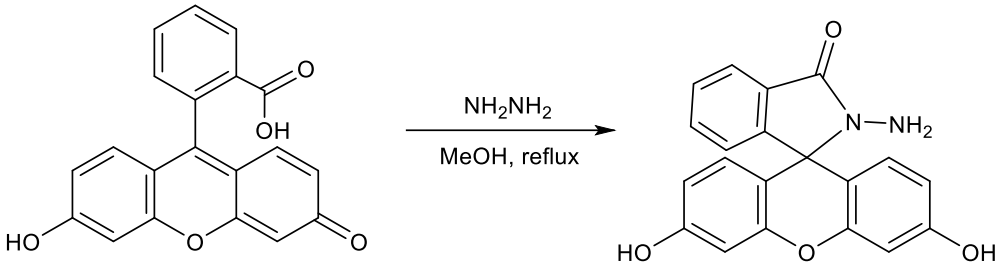
L4

Figure 17. P-gp expression promoter molecule

A glucoconjugate of this compound was proposed in order to improve its poor solubility in water and physiological media, while at the same time promoting its selectivity to the BBB.

7.2 Explored synthetic approaches and results obtained

Firstly, one of the main copper chelator moieties was prepared from the commercially available fluorescein. The desired compound was readily obtained by heterocyclization of fluorescein, as depicted in scheme 11:



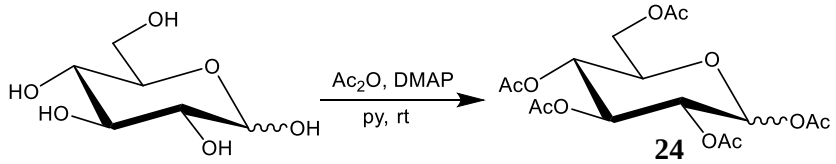
22 **23**

Scheme 11. Synthesis of fluorescein hydrazide (23)

The yields obtained were similar to those given in the literature[251,253] (58%) and compound **20** was purified by chromatography column and/or recrystallization in ethanol.

7.2.1.1 Glucose pentaacetate as a glycosyl donor

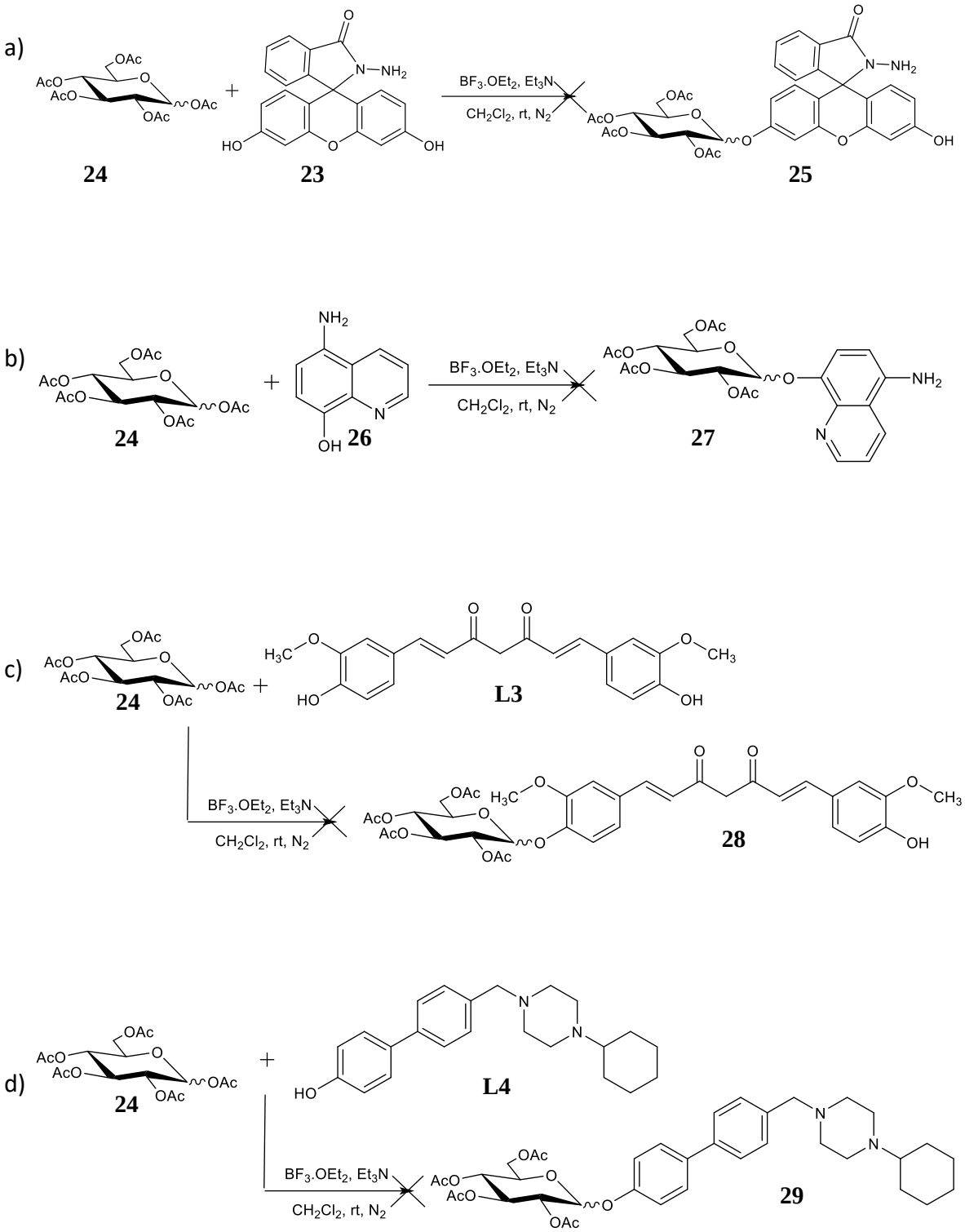
For the preparation of glycosylated analogues, it was necessary to synthesize the glycosyl donor intermediates. The first glycosyl donor synthesized was the 1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose (**24**), which was described as an effective donor in glycoconjugation to phenolic compounds[116,204]. The peracetylation of glucose with acetic anhydride in pyridine in the presence of catalytic amount of dimethylaminopyridine (DMAP) (Scheme 12) is a reaction with a quantitative yield.



24

*Scheme 12. Synthesis of peracetylated glucose (**24**)*

Using this donor, the conjugation to several acceptors was attempted, according to the reactions presented in the scheme 13:

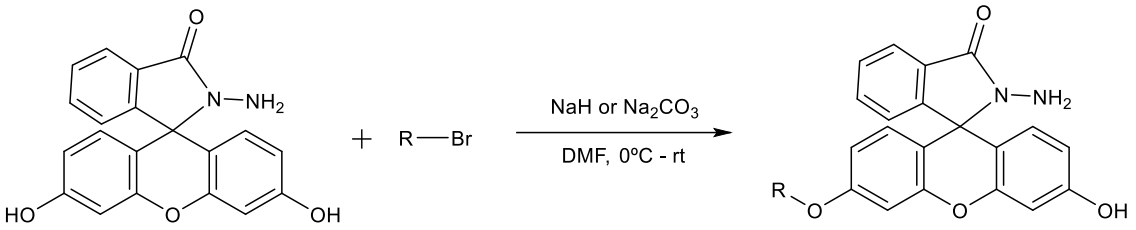


Scheme 13. Glycoconjugation reactions using peracetylglucose as glycosyl donor.

The aglycone chosen for the first reaction (a) is fluorescein hydrazide (**23**). For this type of reaction, since the fluorescein derivative **23** was poorly soluble in dichloromethane (DCM), several reaction conditions were tested, but all the yields obtained were very small (under 7%) or even just traces detectable by MS.

Besides the solubility issue, the low yields were likely associated to the low nucleophilicity of the free OH groups present in the fluorescein derivative. So, in order to improve the likelihood of obtaining a good yield of conjugated derivative, the introduction of a spacer was tested, since this type of conjugation reactions are known to work better for non-aromatic alcohols.[116]

So, the reaction depicted in scheme 14 was tested with a short C_2 chain and a longer C_5 chain:



23

Scheme 14. Synthesis of fluorescein hydrazide with spacer; ***R=CH₂CH₂OH (30)*** *or* ***R=(CH₂)₄CH₂OH (31)***

In both cases, the yields obtained were not good enough to proceed with the glycosylation, but, as expected, the longer chain spacer resulted in a better yield (23%) than the shorter C_2 linker (10%). Attempts of optimization varying the temperature of the reaction to 50˚C and 80˚C produced even lower yields.

For the second aglycone, compound **26** shown in the scheme 11 b), the same reaction system was attempted, expecting that once the aromatic system was smaller, and with higher electronic density available, it would be more nucleophilic, translating into better results for that same reaction system, but, either at room temperature, or when heated to reflux, only a trace amount of the products could be observed after mass analysis.

The synthesis of curcumin glucoconjugates (Scheme 11 c) was attempted under the reaction conditions previously used, but products formed and detected by TLC were not the desired analogues, has shown by mass analysis. Modifications to the protocol showed that the use of $BF_3.OEt_2$ with curcumin resulted in side reactions forming undesirable products, so this reactional system could not be used for this compound.

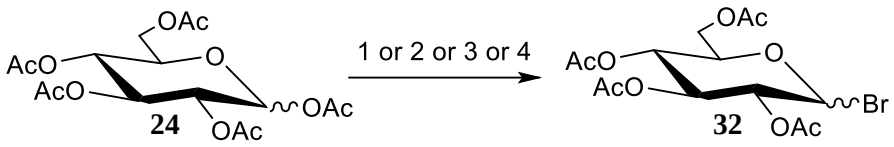
All the previous glucosyl acceptors were moieties capable of chelating copper, but compound **L4,** shown in scheme 11 d), is a P-gp inducer already studied by Biofordrug. Since its solubility in physiological media is very low, a conjugation with glucose would

not only improve its pharmacokinetic properties, but also provide selectivity towards the BBB, where it is desirable to induce the P-gp activity, as discussed before.

The reaction was attempted using the methodology described, but once again, the yields obtained were very low (9%).

7.2.1.2 Employing glucosyl bromide as a glycosyl donor

Since the use of glucose pentaacetate as a glycosyl donor did not produce satisfying results, a different, more reactive donor was prepared according to scheme 13:



*Scheme 15. Preparation of the glucosyl bromide **16** as an intermediate; 1) acetic anhydride, acetic acid, hydrobromic acid (33%)[102]; 2) DCM, acetic anhydride, hydrobromic acid (33%)[260]; 3) hydrobromic acid (33%)[102]; 4) AcBr, AcOH, MeOH*

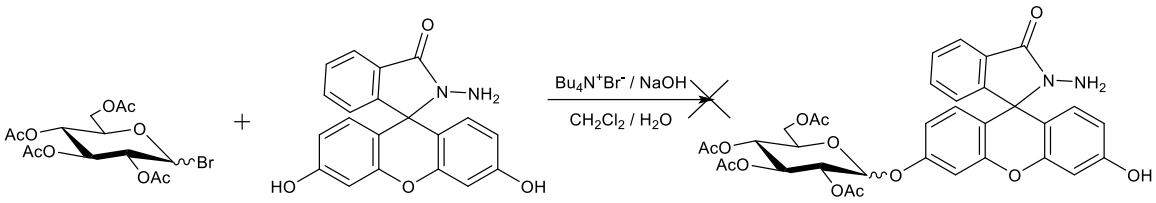
Regarding the anomeric bromination, several methodologies were tested, but only the methodology 4 provided suitable results, as can be seen in table 20. The results registered are not intended to be a methodological comparison, as such disparities can be result of the quality of the hydrobromic acid available.

Table 20. Yields for the glucosyl bromide synthesis

Methodology	**1**	**2**	**3**	**4**
Yield	18%	29%	22%	100%*

*quantitative – by TLC only one product was observed, so the crude was used in the next reaction steps. The other yields were obtained after purification by recrystallization.

From the glycosyl halides used in the aromatic glycoconjugation, glycosyl bromides are by far the most commonly used, providing the best results. In the case of aromatic glycosylation, the reaction system represented in scheme 14 is the most used and provides the best yields:[116]



32 **23** **25**

*Scheme 16. Schematic representation of the glycoconjugation of fluorescein hydrazide (**23**) with glucosyl bromide (**32**)*

This reaction system consists of a phase-transfer system, where a basic aqueous solution is added to an organic phase, ideally containing our reagents, and the presence of a phase transfer catalyst, like tetrabutylammonium bromide.

Attempting this system in the reaction described above (Scheme 14) was not a good option, once the solubility of the fluorescein derivative is very low in DCM, so, only a small quantity was dissolved, and only traces of the desired product were obtained.

7.2.2 Curcumin as a copper chelator and its glucoconjugates

Curcumin is a hydrophobic polyphenol and it has been identified as the active principle of turmeric, the rhizome of the herb *Curcuma longa*, associated with a wide spectrum of biological and pharmacological activities.

Chemically, curcumin is most commonly called diferuloylmethane (Figure 17), which exhibits keto–enol tautomerism having a predominant keto form in acidic and neutral solutions and stable enol form in alkaline medium.

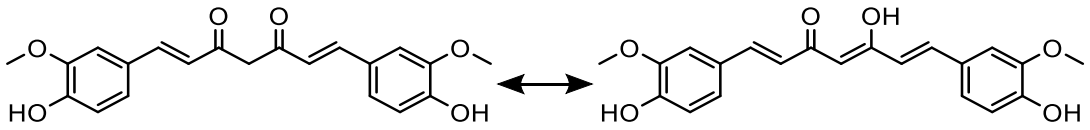


Figure 18. Chemical structure of curcumin and the tautomeric equilibrium

7.2.2.1 Isolation of curcumin

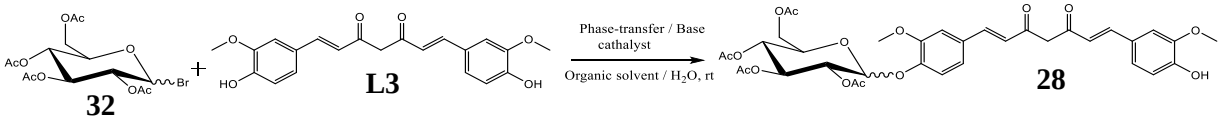
For all the methodologies employed above, the “curcumin” used was not a purified compound, as could be observed by TLC, but probably turmeric, since commercially it is the most common source of curcumin. Do to the unsuccessful results obtained in while using this mixture, extraction and purification of curcumin was performed.

The extraction of curcumin was performed using dichloromethane or acetone as a solvent, where the turmeric powder was suspended, under agitation, for long periods of time (at least 1 hour). It was found that using acetone for the extraction provided better yields. After the desired time, the solvent was filtrated, evaporated, recuperated and added again to the turmeric powder, for at least 4 cycles. Then, curcumin was isolated from this crude extract by chromatographic column. The yield of extraction and purification was 5-10%.

7.2.2.2 Synthesis of curcumin glucosides

For the synthesis of curcumin derivatives, most of the recent literature describes enzymatic or chemoenzymatic approaches[254,255]. Since a purely synthetic approach is preferred, the most widely use method, is a phase-transfer reaction system[256–258], as described for the synthesis using compound **32** as a glycosyl donor.

The general methodology used (Scheme 15) was similar to the one described above, supported by literature[256], which was further modified in an attempt to optimize the results obtained.



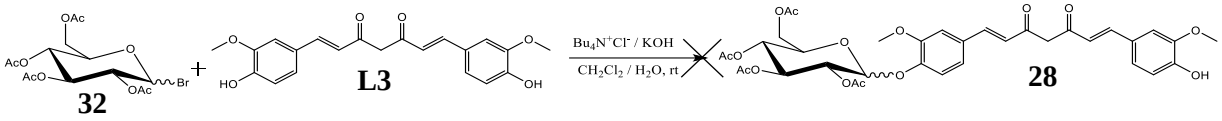
Scheme 17. Schematic representation of the general synthetic pathway

For this system, several phase-transfer catalysts and bases can be used, and since the replication of the reaction conditions observed in the literature did not provide suitable results, several modifications were attempted. These variations are presented in table 21, but no pure compound yields can be presented, since no positive verification of the desired compounds was obtained, after the purification by chromatographic column.

Table 21. Methodological variations applied to the phase-transfer system.

Base	Phase-transfer catalyst	Organic solvent	Temperature
NaOH	$Bu_4N^+Br^-$	CH_2Cl_2	Rt
KOH	$Bu_4N^+Br^-$	CH_2Cl_2	Rt
KOH	$Bu_4N^+Cl^-$	CH_2Cl_2	Rt
Na_2CO_3	$Bu_4N^+Br^-$	CH_2Cl_2	Rt
KOH	$Bu_4N^+Cl^-$	CH_3Cl	Rt
KOH	$Bu_4N^+Cl^-$	CH_2Cl_2	40 ºC

Additionally, better yields have been reported in similar reaction systems that were adapted to microwave-assisted or ultrasound-assisted synthesis[257,259]. With this information, the most promising system, represented in scheme 16, was also used in a microwave assisted synthesis, as well as tested under ultrasound.



Scheme 18. Reaction system submitted to microwave and ultrasound assisted synthesis.

The methodologies here described were chosen based in the good yields presented in the literature as well as the simplicity of the reaction system and simple preparation of the glycosyl donor and acceptor moieties. Unfortunately, the results described in the literature could not be duplicated, but the several optimization steps performed allowed to better understand the reaction system and infer why the desired outcome could not be achieved.

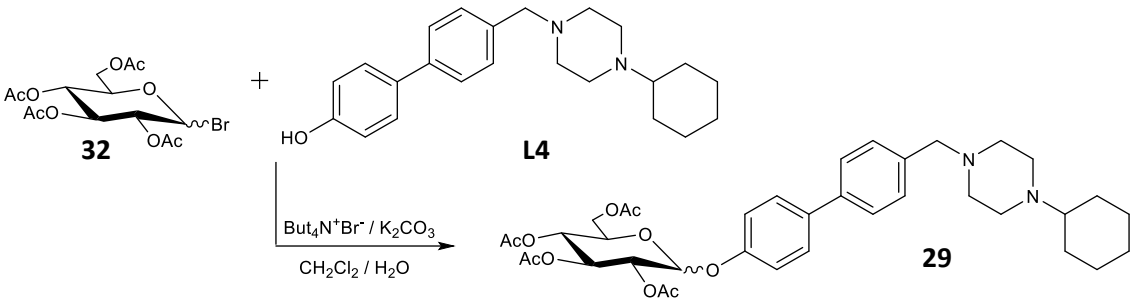
Firstly, an important observation is the chemical sensitivity of curcumin to this reaction system, a fact that it is not mentioned in the literature. Curcumin in the presence of base rapidly starts forming possibly degradation products, as observed by TLC, and it is a favored process to the glycoconjugation. Regardless, curcumin remains the predominant compound, and only after very long reaction times of 72 hours, curcumin could not be observed. Unfortunately, with such a long time of exposure to the reaction mixture, probably most compounds come from decomposition products.

For this reason, increasing the temperature, and using the assisted methodologies should shorten the necessary reaction time, and in this way result in less decomposition products, but what was observed is that decomposition products became the majority compounds in the reaction mixture, even with the shorter reaction times of 5-10 hours.

Another important observation is that even if possible products could be detected by TLC, the attempt of purification by chromatographic column revealed that only small quantities of compound were obtained and none of the compounds tested revealed to be the desired curcumin derivatives, neither mono-glucoside nor di-glucosides.

7.2.3 Synthesis of glucosides of a cyclohexyl piperazine derivative

The phase-transfer system used before was also employed for compound **L4** (Scheme 17), since this compound did not suffer from the stability issues from curcumin. The initial results motivated the optimization of the reactional system, resulting in the reaction conditions used and corresponding yields are presented in table 22.

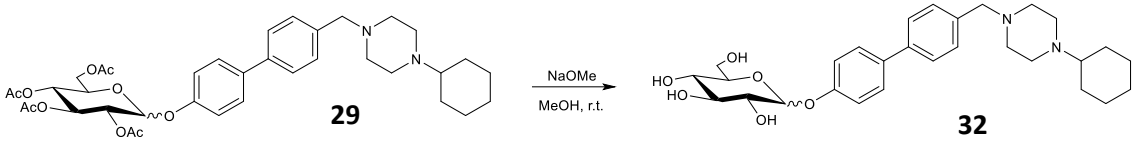


*Scheme 19. Scheme of general synthesis of P-gp inducer **29***

Table 22. Reactional systems and corresponding yields

System nº	Reaction system	Yield
1	$Bu_4N^+F^-$, NaOH, DCM/H_2O, rt	23%
2	$Bu_4N^+Br^-$, NaOH, DCM/H_2O, rt	41%
3	$Bu_4N^+Br^-$, K_2CO_3, DCM/H_2O, rt	47%

The desired product **29** was successfully obtained, proof of the effectiveness of this approach, and we proceeded with the de-acetylation of the glycoconjugate, according to the Zémplen procedure (Scheme 18):



*Scheme 20. Deacetylation of compound **29**.*

This reaction was quantitative, resulting in the desired glycoconjugate capable of being used in biological assays.

Chapter III – General Discussion and Final Remarks

8 General Discussion

The multidisciplinary work presented in this thesis reflects the importance of carbohydrate chemistry and demonstrates its usefulness in multiple branches of life sciences. The contribution given to the development of new antimicrobial compounds, as well as new diagnostic and/or therapeutic options to Alzheimer's disease, praise the relevance of such chemistry as an essential tool for the improvement of biomedicine with subsequent impacts of social relevance.

Hence, this work remains as a personal standpoint expressing my wish for increasing multidisciplinary and cooperative relationships in science. Chemistry provides a unique vision towards modification of available tools, by their adaptation and optimization to address problem-solving approaches, while benefiting greatly from constant feedback as a guideline for further developments. I believe this is especially true in the area of carbohydrate chemistry, due to its ubiquity and intrinsic relation to biological systems and, as such, one of the most beneficial tools for the understanding and tuning of said systems.

The chemistry employed was simple and objective-focused, as an expression of what I take as one of the guidelines of my life: use the simplest tool you can to solve even the most complex problem you have. Complicated and intricate paths should be taken as a requirement but not as an option.

As such, the methodologies employed in the synthesis of the antimicrobial compounds (Section 4.2.2) have proven to be highly efficient and produced the desired results, while allowing further optimization with the development of the one-pot methodology, leading to the products obtained in good overall yields (Section 9.1.4). As a result of this work, important conclusions were taken regarding the importance of some structural features of the alkyl glycosides family, in particular the aglycone structure. Furthermore, these methodologies were also used by our research group for the generation of a small compound library to further establish other essential features to the pursued antimicrobial activity (Section 8.1).

Regarding the design and synthesis of compounds addressing AD, the initial methodology chosen (Section 7.2.1), using pentaacetate glucose as a glycoside donor, was the simplest one reported in the literature, that provided suitable yields. Unfortunately these results could not be translated into the type of compounds under study. Hence, a

more reactive donor, glucosyl bromide (Section 7.2.2), was investigated, leading to much better results. Nonetheless some of the target compounds were not obtained in quantity/purity required to progress to biological studies, especially in the case of curcumin derivatives. In particular the latter, despite extensive literature as proof of suitable methodology and extensive optimization attempts, no suitable products were identified. But, as an example of the suitability of the chosen methodology, the P-gp expression promoter (compound **32**), was successfully synthesized.

The work developed at Biofordrug constituted an innovation in the research performed in the company, with the first incorporation of sugar moieties leading to biocompatible candidates, improving the properties of previously studied molecules, and establishing important methodological guidelines.

8.1 Important structural features of alkyl glycosides

The joint synthetic effort of several members of the research group allowed for the generation of a great diversity of structural analogues to the starting scaffold, compound **1**. The study of bacterial susceptibility to this small compound library made it possible to establish a relationship between the different structural features and their impact in compounds' antimicrobial activity, as summarized in figure 18.

Two main structural groups were modified separately: i) the alkyl chain that constitutes the most lipophilic part of the molecule and which modification was the main focus of the presented work; ii) the sugar moiety, the most hydrophilic portion of the molecule. Both are essential to the amphiphilic character of the studied compounds, and so both will be discussed.

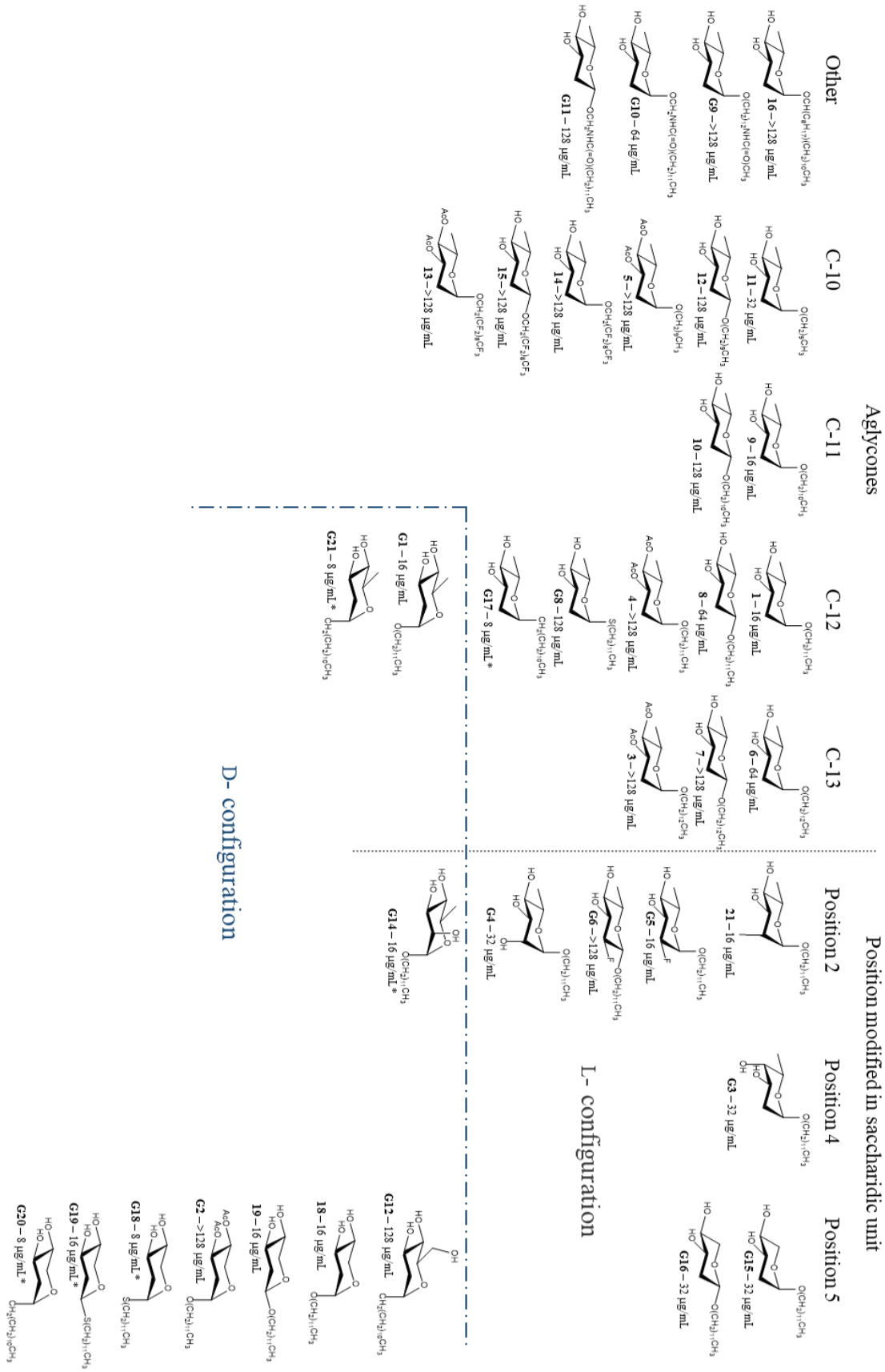


*Figure 19. Visual summary of the structural diversity of compounds obtained and their corresponding bioactivity. Label: number of compound : MIC value; *MIC value obtained in NIRJ (Table 9); compounds labelled G were obtained by other group members*

As expected from the literature analysis (Section 4.1), the aliphatic chain is one of the key factors to the biological activity under study, since any modifications to its single saturated chain or its lipidic character showed a decrease in biological activity, observed by comparing the bioactivity of compounds **14**, **16**, **G9** and **G10** with that of compound **1**, probably due to the need of good membrane intercalating properties. The length of the aliphatic chain is also a crucial factor to bioactivity, with the ideal number of carbon atoms being 12. By changing this number, a decrease in antibacterial activity has been observed, as shown when comparing compounds **6**, **9** and **11** to **1**, and/or the respective β anomers (**7**, **10** and **12** to **8**). These results are in agreement with data given in the literature, as referred in section 4.1.

Modifications to the lipophilicity of the alkyl chain were also tested. By comparing compounds **11** to **14** and **16** to **1**, we can conclude that its decrease or increase leads to inactive compounds, possibly due to the modification of the intercalating capabilities of these alkyl glycosides, resulting in an ineffective interaction with the bacterial membrane.

Finally, attempts at functionalizing the alkyl chain showed that any modification of the amphiphilic character leads to less active analogues (**G10**, comparing to **1**) and the more drastic the modification, the less active the compound, with compound **G9** representing the extreme case of introduction of a polar group in the aliphatic chain terminal.

As a result of the analysis above described, the C_{12} alkyl chain was deemed essential and optimal to the bioactivity observed and a non-functionalizable portion of the molecule.

Analogues where the glycosidic bond was modified (compound **1** vs **G5** or **G8** and **18** vs **G18** or **G20**) show that the atom linking the dodecyl chain to the sugar induces a decrease in biological activity in the case of the sulphur atom (C-O vs C-S bond) but showed no significant alteration of the MIC values when linked by a carbon atom (C-O vs C-C bond).

Regarding the study of the sugar moiety, the hydrophilic portion of these molecular structures revealed a further potential to optimization and functionalization. Firstly, demonstrating the importance of the hydrophilic character and the polarity of the sugar moiety, when the hydroxy groups are protected by acetyl groups, all the bioactivity

observed is lost as observed by comparing compounds **1** and **4**, **5** and **11**, **18** and **G2** and even **13** and **14** (this last is a special case to be further discussed).

Structurally speaking, the deoxygenation pattern of such compounds was a cornerstone for the development of this family of glycosides, and, as such, its investigation was essential.

Considering the deoxygenation at the 6-position, it was found to be essential to the biological activity observed, by comparing compounds **G12** and **G21**, but also when the sugar moiety is a xyloside derivative, devoid of this position, a slight decrease in bacterial activity was observed, by comparing compound **1** with **G15**, but not detected between compounds **G1** and **18**, although compound **1** was shown to be more effective than compound **18** (Section 6.1.2).

The deoxygenation at the 2-position was shown not to be essential to the bioactivity, since compounds **G4** and **G14** retained some bioactivity although less than the corresponding 2-deoxy analogues (compounds **1** and **G1**). On the other hand, a halogen substituent in that position gives rise to compounds showing bioactivity similar to their non-halogenated derivatives (compound **1** vs **21** and **G5**).

Regarding the remaining hydroxy groups, when comparing compound **1** and **G3**, it can be mentioned that chirality center configuration is important to the bioactivity, being preferential the configuration with the substituent in. equatorial position

Comparing compounds **1** and **G1** (as well as **18** and **G15**), we can infer that the D- and L- configurations do not have significant impact in the bioactivity of these alkyl glycosides, although the xyloside derivative shows preference for the D- configuration.

Comparison of the bioactivity of α/β pairs provided some interesting and unexpected results. The MIC values of compound pairs **1** and **8**, **6** and **7**, **9** and **10**, **11** and **12** show that a major difference in bioactivity is present with at least a 4-fold increase in MIC values for the β-anomer. This is not in accordance to the literature data and previously described (Section 4.1), where α/β pairs showed very similar bioactivity, normally within a 2-fold difference. Equally interesting is the fact that when we analyse the xyloside derivatives (compounds **18** and **19**, **G15** and **G16**, **G18** and **G19**), this difference is no longer present, and the compounds behave much more in accordance to the literature, showing no biologically relevant difference in bioactivity.

Concluding this analysis, we have established the optimal structural features to the antimicrobial activity of this group of compounds, with compound **1** containing all these essential features, while allowing the further functionalization of its position-2.

Noteworthily, one of the structural modifications resulted in some unexpected unrelated biological effects. Compound **14** has shown no antimicrobial effects and, actually, has demonstrated an opposing effect, causing an increase in the bacterial population in stationary phase (Annex 4). This unexpected biological effect is not observed in compound **13**, showing that it depends on the presence of free hydroxy groups and possibly on the specific amphiphilic character of this molecule. Although similar potentiating effects can be observed in bacterial growth under stress conditions, such effects are normally not so pronounced (compound **14** caused a 30% increase in bacterial growth) and also cause a lag phase in bacterial growth, what was not observed for this compound. Nonetheless, since such effects were not the focus of the present study and no supporting literature to such effects was found, a new and extensive research effort was not initiated.

8.2 The profile of biological activity

From the bacterial growth curves (annex 3) obtained for all the compounds tested by the microdilution methodology we observed that the behavior, or bacterial response, demonstrated to all the biologically active compounds was of a similar profile to that observed for compound **1**, with no noteworthy differences such as much shorter or longer action times, lag phases or other bacterial growth characteristics. This fact can be considered as a strong indicator that the mechanism of action of these compounds is the same or closely related.

Additionally, the fact that MIC and MBC values are equal or similar in all the compounds tested (Tables 7 to 9) indicates that the observed biological activity is bactericidal in nature and not bacteriostatic, or, in the case of two distinct mechanisms of action being present, the bactericidal action is predominant to the bacteriostatic activity. Also, this bactericidal action was shown to be very fast, at least for compounds tested by time-kill assays **1** and **18** (Section 6.1.1). This fast-acting bactericidal effect is one of the main goals in the development of new antimicrobial compounds, since it reduces the time necessary for a viable bacterial population to be exposed to the antimicrobial compound until its complete destruction, leaving less time to phenotypic adaptations and other resistance-conferring strategies.

While a correlation between many structural features and the antimicrobial activity of these compounds was demonstrated, the same correlation was not observed with the toxicity of studied compounds (Section 4.3.1). The cytotoxicity of all compounds was very similar (within a $\log_2$ variability of IC_{50} values), even though some structures showed remarkable differences in bioactivity (compound **1** compared to **G8**). Nonetheless, a tendency of *C*-glycosides to exhibit less toxicity without a significant difference in antimicrobial activity was observed. Such observations are an important indication that the mechanism of antibacterial activity is diverse from that of cytotoxicity.

8.3 Alkyl glycosides, an answer to a problem?

Several studies were performed aimed at evaluating the applicability of these compounds to uses granted to compounds of a similar structure (Section 5). Firstly, one of the most important results of such studies was the assessment of the capability of these compounds to counteract the pressing resistance problem (Section 5.1). Here we showed

that not only the mechanism of action of these compounds is unrelated to any of the main classes of antibiotics used, and so no cross-resistance effects are to be expected, as bacteria exhibiting resistance to all the denoted classes have remained susceptible to the action of compound **1** with no detectable variation in MIC values. More important is the fact that even after extensive exposure to compound **1**, the bacterial culture was unable to acquire any measurable resistance to the action of this compound, overcoming many of the major hurdles in current antibiotic therapy as discussed in section 1.1.

Since other similar compounds are applied in surface cleaning and disinfection, studies of similar application were performed, but with unexpectedly poor results. As later shown, this was probably due to their ineffectiveness against bacterial spores, consequence of their inability to overcome the thick spore coat. Possibly such issues could be overcome in a formulation with other sporicidal agents, which is probably what enables the application of other similar compounds to the ends above mentioned.

8.3.1 Peculiarities of the mechanism of action

The decision to study the mechanism of action of these compounds besides the expected surfactant action was promoted by a number of factors associated to the disparities between the available literature and what was observed for the studied compounds. Examples of this are the difference between the bioactivity of α and β anomers and the lower MIC values obtained as well as the apparent selectivity to bacteria of the *Bacillus* genus.

After the determination and characterization of the bactericidal activity observed for compound **1**, evaluation of its impact in bacterial viability revealed the previously undetected presence of a persister sub-population. Bacterial vitality studies have confirmed this but have also shown that the persister population resulting from the very fast reduction of metabolic active bacteria caused by the action of compound **1** was also declining with time, a very important factor to take into account, since compounds capable of acting in persister populations are in demand once, this population represents one of the major drivers of resistance (Section 1.1).

The search for a possible metabolic target for compound **1** was initiated by a differential metabolomic study (Section 6.1.4) and the diversification of biological effects observed, dependent on different carbon sources, was a major factor considering possible molecular targets, since such variation of results was not expected in the case of a non-

specific surfactant effect. The analysis of the results obtained lead to the creation of a list of possible targets, but, unfortunately, upon target evaluation by creation of mutant variants and their susceptibility, no positive target identification was achieved. Nonetheless, a target in common with all results obtained in the metabolic analysis emerged as a suitable alternative, the bacterial membrane.

After the assessment of compound **1** activity against active and resistant bacterial species and their spheroplast and protoplasts we were able to show that the main factor leading the inactivity of such compounds to Gram-negative bacteria is the outer bacterial membrane (at least in the case of *E. coli* K12), and more importantly, that the unexpected specificity of this compound to *Bacillus spp.* was probably associated to the bacterial membrane composition, since the resistance observed of *S. aureus* ATCC 29213 remained unaltered, with only its membrane exposed to the activity of compound **1**. This was one of the most surprising results obtained, since no similar examples of such specificity exist in the literature for structurally related compounds. If such selectivity could be fine-tuned these compounds could be of a major therapeutic advantage for the reasons already explained in length in section 1.2.1.4. The activity over the bacterial membrane was finally verified using AFM and registering all the morphologic modifications associated to membrane damage induction and permeabilization, even at sub-inhibitory concentrations.

8.4 Final remarks

The objective of the work performed was the determination or better understanding of the mechanism of action for the family of compounds and this was achieved. Our compounds demonstrated some characteristics not found in the literature for alkyl glycosides, namely their apparent selectivity. This phenomenon was then further studied by computational and fluorescence studies, trying to validate a hypothesis: the selectivity towards a type of lipid composition in the bacterial membrane.

From the results obtained we could conclude that the mechanisms involved in these interactions were indeed most relevant in the presence of neutral lipids in the bacterial membrane, as were found to be the ones most prone to suffer lipid phase transitions when exposed to the compound in study.

Such results could explain the ineffectiveness of these compounds regarding the *S. aureus* spp. while having notable activity against the *E. coli* spheroplasts, showing that its activity is not due to the Gram-positive/Gram-negative metabolic differences, but due to their physical barriers and membrane composition.

When addressing the mechanism of action, various results point to a mechanism devoid of molecular target and associated to the referred interaction of a physical chemical nature with the cytoplasmic membrane, leading to a destabilization and consequently to cell lysis.

With the high number of compounds created and tested, we were able to derive some structure-activity relationships for these types of compounds, granting us the capacity to understand the impact of further modifications in its biological activity. It was also observed that all the tested compounds, generally, follow the same bactericidal action profile, being defined as a family of compounds with the same mechanism of action.

Furthermore, the methodologies developed have proven to be of great value and consisted of a relatively quick pathway to characterize and provide important insights on the mechanism of action of any type of compounds, since their application was totally devoid of any specificity to the compound, once no labeling was used nor necessary.

These aspects constitute a great increase in the knowledge available in the literature, not only for these types of compounds, to which not much biological relevance was given until now, as well as methodologically, since the highly interdisciplinary work

performed showed that, with proper coordination of efforts, we could largely increase the amount of biologically relevant compounds with previously unknown mechanisms of action, since most of the methodologies applied are translatable to other fields of biological studies.

To further develop the studies here presented, the focus should be the better understanding of the physicochemical processes that govern the interaction of these compounds with biological membranes, with special focus in selective lipid interaction.

At the same time, to further explore the selectivity of these compounds, it is important to expand the variety of bacterial species to test the susceptibility of compound **1**. The relevance of this study is associated with the fact that lipidic composition of the bacterial membranes varies greatly between bacterial species.

With this in mind, it would be of great importance the susceptibility assessment not only of their vegetative cells, but also of the respective spheroplasts and protoplasts, since it would be the most direct assessment of the compounds action on the bacterial membrane.

The studies of these interactions should also be continued using model lipid bilayers, since their composition can be highly controlled, even if certain structures or organizations present in biological membranes are lacking. A similar approach by computational methods could also be of importance to the further understanding of this phenomena.

Chapter IV – Experimental Section

9 Experimental section

9.1 Synthesis of antimicrobials

9.1.1 General methods and materials

Starting materials and reagents were purchased from Sigma–Aldrich, Fluka and Acros. Melting points were determined on an Stuart melting point SPM30 apparatus. 1H and 13C NMR spectra as well as additional correlation spectroscopy (COSY and HMQC) were obtained on a BRUKER Avance 400 (400.13 MHz for 1H and 100.62 MHz for 13C) apparatus for solutions in deuterated acetone (D6) or chloroform (CDCl3) (1% v/v Me4Si, Sigma-Aldrich), at room temperature, using tetramethylsilane as an internal reference. Chemical shifts are reported in δ (ppm). The values of specific rotation were measured in a Perkin Elmer Polarimeter 343, with a temperature stabilizer from P Selecta, for solutions in CH_2Cl_2.

Reagents for synthetic procedures were purchased to Sigma-Aldrich and the reactions performed were controlled by Thin-Layer Chromatography (TLC), using aluminum plates with a coating of silica gel Alugram Xtra Sil G/UV F254 (Macherey-Nagel). The detection of products in TLC was made by observation under UV light (254 nm) (Camag) and/or by spraying the plate with a solution of sulfuric acid in pure ethanol 10% (v/v) and heated to 120 ºC with a hot-air pistol. The verification of the neutralization processes was made using pH paper (Filterlab, RL911R07).

The solutions were concentrated in a rotary evaporator (Buchi.V220) at 40 ºC under reduced pressure. The separation and purification of products was made by column chromatography, using silica gel 60 G (0.040-0.063 mm, SDS). The quantity of stationary phase to use was approximately 50 times the mass of the dry residue. All the solvents used (Carlo Erba, VWR, Panreac) were previously distilled and maintained in molecular sieves 4 Å. Glassware was previously dried in a stove at 100 ºC.

9.1.2 General procedures for glycal donor synthesis

9.1.2.1 Glycal synthesis

To a solution of the commercial free sugar (6 mmol) in pyridine (10 mL/g), acetic anhydride (2 eq./OH, 4.5 mL, 48 mmol) was slowly added and, finally, a catalytic amount of DMAP (ca. 20 mg). Reaction mixture was left to stir at room temperature for two hours. Then, the solution was diluted with dichloromethane (30 mL) and washed 3 times with HCl 2M and finally with brine. The organic phase was dried with anhydrous $MgSO_4$, filtered and concentrated under vacuum, affording the peracetylated sugar as a colourless oil. This reaction provided quantitative yields of pure products, as verified by TLC and NMR, without further purification being necessary.

Method 1: The resulting product was dissolved in glacial acetic acid (5 mL/g) to which acetic anhydride (0.9 mL, 9 mmol, 1.5 eq.) and hydrobromic acid (33%) (3 mL, 0,018 mmol, 3 eq.) were added. Reaction mixture was kept stirring at room temperature protected from light and under nitrogen. After 3h, reaction mixture was diluted with dichloromethane and washed 3 times with saturated $NaHCO_3$ and with brine. The organic phase was dried with anhydrous $MgSO_4$, filtered and concentrated under vacuum, affording the corresponding glycosyl bromide as a translucent yellow oil.

Method 2: The resulting product was dissolved in glacial acetic acid (5 mL/g) to which methanol (0.4 mL, 9 mmol, 1.5 eq.) and acetyl bromide (1.4 mL, 0,018 mmol, 3 eq.) were added. Reaction mixture was kept stirring at room temperature protected from light and under nitrogen. After 3h, reaction mixture was diluted with dichloromethane and washed 3 times with saturated $NaHCO_3$ and with brine. Organic phase was dried with anhydrous $MgSO_4$, filtered and concentrated under vacuum, affording the corresponding glycosyl bromide as a translucent yellow oil.

The obtained glycosyl bromide was dissolved in acetone (30 mL, 5 mL/mmol), sodium dihydrogen phosphate (12.8 g, 132 mmol, 22 eq.) and zinc (5.9 g, 90 mmol, 15 eq.) were added and mechanically stirred vigorously, at room temperature. After 10 min, water (3 mL, 0.5 mL/mmol) was also added and kept stirring for 1h30. Reaction mixture was extracted with ethyl acetate, washed twice with saturated $NaHCO_3$ and then brine. Organic phase was dried with anhydrous $MgSO_4$, filtered and concentrated under vacuum. The resulting residue was purified with column chromatography with Hexane/EtOAc, affording the corresponding glycal.

3,4-Di-*O*-acetyl-1,5-anhydro-2,6-dideoxy-L-*arabino*-hex-1-enitol (2). Obtained from L-rhamnose (5.0 g, 30 mmol) as a colourless oil, in 39 % yield according to method 1 (2.5 g, 12 mmol) and 74 % yield according to method 2 (4.8 g, 22 mmol). R_f =0.31 (EtOAc/Hexane 1:5). **^{1}H RMN** ($CDCl_3$) δ 6.43 (br d, 1H, $J_{1,2}$ = 6.04 Hz, H-1), 5.33 (m, 1H, H-3), 5.02 (dd, 1H, $J_{3,4}$=6.22 Hz, $J_{4,5}$=8.06 Hz, H-4), 4.77 (dd, 1H, $J_{2,3}$= 2.98 Hz, H-2), 4.10 (quint, 1H, $J_{4,5}$= $J_{5,6}$=6.93 Hz, H-5), 2.08 (s, 3H, -OAc), 2.04 (s, 3H, -OAc), 1.31 (d, 3H, H-6).

9.1.3 Procedures for the glycoconjugation reaction and deprotection

*9.1.3.1 General procedure for the synthesis of L-*arabino*-glycosides*

A solution of the alkyl nucleophile (4,668 mmol, 2 eq.) and TPHB (86,55 mg, 0.252 mmol, 0.11 eq.) in dry CH_2Cl_2 (3.0 mL) (or CH_3CN for compounds **13-15**) was added to a solution of 3,4-di-*O*-acetyl-6-deoxy-L-glucal **(2)** (500 mg, 2.334 mmol) in dry CH_2Cl_2 (3.0 mL). The mixture was stirred at room temperature for 50 min. After, CH_2Cl_2 (6 mL) was added to the reaction mixture and the solution washed with a saturated solution of $NaHCO_3$ (for compounds 3,9,10, basic Amberlite IRA-400 is added and the solution is filtered). The organic phase is dried with anhydrous $MgSO_4$, filtered, the solvent evaporated and column chromatography with EtOAc/n-hexane afforded the two anomers of the corresponding 2,6-dideoxy glycosides as well as the Ferrier compound as a secondary product.

Tridecyl 3,4-di-*O*-acetyl-2,6-dideoxy-α-L-*arabino*-hexopyranoside (3)

Using the above described protocol, with tridecanol (935 mg, 4.67 mmol) as the nucleophile, gave **3** as a white solid (551 mg, 57%); Rf 0.56 (1:5 EtOAc/n-hexane);

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.30 (ddd, 1H, H-3, $J_{3,2eq}$ = 5.35 Hz, $J_{3,2ax}$ = 11.42 Hz, $J_{3,4}$ = 9.65 Hz), 4.87 (d, 1H, H-1, $J_{1,2ax}$ = 2.80 Hz), 4.75 (t, 1H, H-4, $J_{4,3}$ = $J_{4,5}$ = 9.60 Hz), 3.87 (dq, 1H, H-5, $J_{5,6}$ = 6.26 Hz), 3.62 (dt, 1H, H-1'a, $J_{1'a,1'b}$ = 9.35 Hz, $J_{1'a,2'}$ = 6.85 Hz), 3.36 (dt, 1H, H-1'b, $J_{1'b,2'}$ = 6.80 Hz), 2.23 (ddd, 1H, H-2eq, $J_{2eq,3}$ = 5.30 Hz, $J_{2eq,2ax}$ = 12.88 Hz, $J_{2eq,1}$ = 1.01 Hz), 2.07 (s, 3H, CH_3CO$_2$), 2.03 (s, 3H, CH_3CO$_2$), 1.80 (td, 1H, H-2ax, $J_{2ax,2eq}$ = $J_{2ax,3}$ = 12.29 Hz, $J_{2eq,3}$ = 3.37 Hz), 1.61 (br. s, 2H, H-2'), 1.38-1.23 (m, 14H, H-3'-H-12'), 1.19 (d, 3H, H-6, $J_{6,5}$ = 6.26 Hz), 0.90 (t, 3H, H-13', $J_{10',9'}$ = 6.68 Hz)

ppm. ^{13}C NMR ($CDCl_3$) δ = 170.3 (C=O, OAc), 96.6 (C-1), 75.0 (C-4), 69.2 (C-3), 67.6 (C-1'), 65.4 (C-5), 35.4 (C-2), 31.9, 29.7, 29.6, 29.5, 29.5, 29.4, 29.4, 26.2, 22.7 (C-2' to C-12'), 21.1, 20.9 (CH_3, -OAc), 17.6 (C-6), 14.1 (C-13').

Dodecyl 3,4-di-*O*-acetyl-2,6-dideoxy-α-L-*arabino*-hexopyranoside (4)

Using the above described protocol, with dodecanol (870 mg, 4.67 mmol) as the nucleophile, gave **4** as a white solid (552 mg, 59%); Rf 0.54 (1:5 EtOAc/n-hexane);

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.21 (ddd, 1H, H-3, $J_{3,2eq}$ = 5.41 Hz, $J_{3,2ax}$ = 11.61 Hz, $J_{3,4}$ = 9.60 Hz), 4.77 (d, 1H, H-1, $J_{1,2ax}$ = 3.20 Hz), 4.65 (t, 1H, H-4, $J_{4,3}$ = $J_{4,5}$ = 9.60 Hz), 3.78 (dq, 1H, H-5, $J_{5,6}$ = 6.26 Hz), 3.53 (dt, 1H, H-1'a, $J_{1'a,1'b}$ = 9.58 Hz, $J_{1'a,2'}$ = 6.75 Hz), 3.28 (dt, 1H, H-1'b, $J_{1'b,2'}$ = 6.65 Hz), 2.14 (ddd, 1H, H-2eq, $J_{2eq,3}$ = 5.43 Hz, $J_{2eq,2ax}$ = 12.77 Hz, $J_{2eq,1}$ = 1.01 Hz), 1.98 (s, 3H, CH_3CO$_2$), 1.93 (s, 3H, CH_3CO$_2$), 1.70 (ddd, 1H, H-2ax, $J_{2ax,2eq}$ = 12.66 Hz, $J_{2ax,3}$ = 11.78 Hz, $J_{2eq,3}$ = 3.68 Hz), 1.49 (br. s, 2H, H-2'), 1.27-1.16 (m, 14H, H-3'-H-11'), 1.10 (d, 3H, H-6, $J_{6,5}$ = 6.28 Hz), 0.81 (t, 3H, H-12', $J_{10',9'}$ = 6.82 Hz) ppm. ^{13}C NMR ($CDCl_3$) δ = 170.3 (C=O, OAc), 96.7 (C-1), 74.5 (C-4), 69.0 (C-3), 68.0 (C-1'), 65.5 (C-5), 35.5 (C-2), 30.6, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 26.2, 25.9, 22.9 (C-2' to C-11'), 21.1, 20.9 (CH_3, -OAc), 17.6 (C-6), 14.1 (C-12').

Decyl 3,4-di-*O*-acetyl-2,6-dideoxy-α-L-*arabino*-hexopyranoside (5)

Using the above described protocol, with decanol (740 mg, 4,67 mmol) as the nucleophile, gave **5** as a white – pale yellowish solid (529 mg, 61%);

^{1}H NMR (400 MHz, CDCl3): δ = 4.75 (ddd, 1H, H-3, $J_{3,2eq}$ = 5.46 Hz, $J_{3,2ax}$ = 11.50 Hz, $J_{3,4}$ = 9.63 Hz), 4.85 (d, 1H, H-1, $J_{1,2}$ = 3.85 Hz), 4.73 (t, 1H, H-4, $J_{4,3}$ = $J_{4,5}$ = 9.60 Hz), 3.85 (dq, 1H, H-5, $J_{5,6}$ = 6.30 Hz), 3.60 (dt, 1H, H-1'a, $J_{1'a,1'b}$ = 9.47 Hz, $J_{1'a,2'}$ = 6.76 Hz), 3.35 (dt, 1H, H-1'b, $J_{1'b,2'}$ = 6.57 Hz), 2.21 (dd, 1H, H-2eq, $J_{2eq,3}$ = 5.31 Hz, $J_{2eq,2ax}$ = 12.63 Hz), 2.05 (s, 3H, CH_3CO$_2$), 2.01 (s, 3H, CH_3CO$_2$), 1.78 (td, 1H, H-2ax, $J_{2ax,2eq}$ = $J_{2ax,3}$ = 12.19 Hz, $J_{2eq,3}$ = 3.66 Hz), 1.57 (m, 2H, H-2'), 1.31 (m, 14H, H-3'-H-9'), 1.17 (d, 3H, H-6, $J_{6,5}$ = 6.30 Hz), 0.88 (t, 3H, H-10', $J_{10',9'}$ = 6.69 Hz) ppm. ^{13}C NMR ($CDCl_3$) δ = 170.3 (C=O, OAc), 96.6 (C-1), 75.0 (C-4), 69.2 (C-3), 67.6 (C-1'), 65.4 (C-5), 35.4 (C-2), 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 26.2, 22.7 (C-2' to C-9'), 21.1, 20.9 (CH_3, -OAc), 17.6 (C-6), 14.1 (C-10').

1H,1H Perfluorodecyl 3,4-di-*O*-acetyl-2,6-dideoxy-α-L-*arabino*-hexopyranoside (13) Using the above described protocol, with 1H,1H Perfluorodecanol (2,33 g, 4,67 mmol) as a nucleophile, gave **13** as a white solid (838 mg, 50%);

^{1}H NMR (400 MHz, CDCl3): δ = 5.25 (dt, 1H, H-3, $J_{3,2ax}$ = 10.48 Hz, $J_{3,2eq}$ = 5.43 Hz), 4.91 (d, 1H, H-1, $J_{1,2ax}$ = 3.28 Hz), 4.77 (t, 1H, H-4, $J_{4,3} = J_{4,5}$ = 9.60 Hz,), 4.07 (q, 1H, H-1'a, $J_{1'a,1'b} = J_{1'a,F2'a} = J_{1'a,F2'b}$ = 13.51 Hz), 3.97 (q, 1H, H-1'b, $J_{1'b,1'a} = J_{1'b,F2'a} = J_{1'b,F2'b}$ = 13.51 Hz), 3.85 (dq, 1H, H-5), 2.34 (dd, 1H, H-2eq, $J_{2eq,2ax}$ = 13.26 Hz, $J_{2eq,3}$ = 5.43 Hz), 2.07 (s, 3H, CH_3CO$_2$), 2.02 (s, 3H, CH_3CO$_2$), 1.84 (dt, 1H, H-2ax), 1.20 (d, 1H, H-6, $J_{6,5}$ =6,32) ppm.

9.1.3.2 General procedure for the deacetylation reaction

A solution of NaOMe in MeOH (1%, 10 mL) was added to a solution of the acetylated sugar (1.3 mmol) in MeOH (60 mL) and the mixture was stirred at rt for 1 h. Neutralization with Amberlite (IR-120) was followed by filtration and evaporation of the solvent, and the resulting residue was submitted to purification by chromatographic column, eluted with 1:1 EtOAc–n-hexane affording the corresponding 2,6-dideoxy glycosides.

Dodecyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside (1)

Using the above described protocol for the anomeric mixture (503 mg), gave **1** as a white solid (242 mg, 61%);

^{1}H NMR (400 MHz, CDCl3): δ = 4.83 (d, 1H, H-1, $J_{1,2}$ = 3.03 Hz), 3.93 (ddd, 1H, H-3, $J_{3,2eq}$ = 5.05 Hz, $J_{3,2ax}$ = 11.56 Hz, $J_{3,4}$ = 8.91 Hz), 3.70-3.57 (m, 2H, H-5, H-1'a), 3.35 (dt, 1H, H-1'b, $J_{1'b,1'a}$ = 9.41 Hz, $J_{1'b,2}$ = 6.51 Hz), 3.11 (t, 1H, H-4, $J_{4,3} = J_{4,5}$ = 9.09 Hz), 2.13 (dd, 1H, H-2eq, $J_{2eq,2ax}$ = 12.76 Hz), 1.69 (td, 1H, H-2ax, $J_{2ax,1}$ = 3.66 Hz), 1.61-1.52 (m, 2H, H-2'a, H-2'b), 1.41-1.21 (m, 20H, H-6, H-3'-H-9'), 0.89 (t, 3H, H-10', $J_{10',9'}$ = 6.69 Hz) 13C NMR (100 MHz, CDCl3): δ = 97.2 (C-1), 78.1 (C-4), 69.3 (C-5), 67.4 (C-1', C-5), 37.9 (C-2), 31.9 (C-1'), [29.6, 29.5, 29.5, 29.4, 29.3, 26.2, 22.7] (C-3'-C-11'), 17.7 (C-6), 14.1 (C-12') ppm

Dodecyl 2,6-dideoxy-β-L-*arabino*-hexopyranoside (8)

Using the above described protocol for the anomeric mixture (503 mg), gave **8** as a white solid (103 mg, 26%);

^{1}H NMR (400 MHz, CDCl3): δ = 4.49 (dd, 1H, H-1, $J_{1,2ax}$ = 9.60 Hz, $J_{1,2eq}$ = 1.77 Hz), 3.87 (dt, 1H, H-1'a, $J_{1'a,2'}$ = 6.82 Hz, $J_{1'a,1'b}$ = 9.35 Hz), 3.62 (ddd, 1H, H-3,), 3.28 (dq, 1H, H-5, $J_{5,4}$ = 8.83 Hz), 3.12 (t, 1H, H-4, $J_{4,3}$ = $J_{4,5}$ = 8.83 Hz), 2.23 (ddd, 1H, H-2eq, $J_{2eq,2ax}$ = 12.44 Hz, $J_{2eq,3}$ = 4.99 Hz), 1.68-1.54 (m, 3H, H-2ax, H-2'a, H-2'b), 1.40-1.21 (m, 21H, H-6, H-3'-H-11'), 0.88 (t, 3H, H-10', $J_{10',9'}$ = 6.82 Hz) ppm.

Decyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside (11)

Using the above described protocol for the anomeric mixture (506 mg), gave **11** as syrup (267 mg, 68%);

^{1}H NMR (400 MHz, CDCl3): δ = 4.75 (d, 1H, H-1, $J_{1,2}$ = 3.03 Hz), 3.84 (ddd, 1H, H-3, $J_{3,2eq}$ = 5.05 Hz, $J_{3,2ax}$ = 12.10 Hz, $J_{3,4}$ = 9.09 Hz), 3.61-3.49 (m, 2H, H-5, H-1'a), 3.27 (dt, 1H, H-1'b, $J_{1'b,1'a}$ = 9.41 Hz, $J_{1'b,2}$ = 6.59 Hz), 3.03 (t, 1H, H-4, $J_{4,3}$ = $J_{4,5}$ = 9.09 Hz), 2.05 (dd, 1H, H-2eq, $J_{2eq,2ax}$ = 12.76 Hz), 1.60 (td, 1H, H-2ax, $J_{2ax,1}$ = 3.50 Hz), 1.54-1.43 (m, 2H, H-2'a, H-2'b), 1.40-1.14 (m, 17H, H-6, H-3'-H-9'), 0.81 (t, 3H, H-10', $J_{10',9'}$ = 7.04 Hz) ppm. 13C NMR (100 MHz, CDCl3): δ = 97.2 (C-1), 78.1 (C-4), 69.3 (C-5), 67.4 (C-1'), 67.3 (C-5), 37.9 (C-2), 31.9 (C-1'), [29.6, 29.5, 29.4, 29.3, 26.2, 22.7] (C-3'-C-9'), 17.7 (C-6), 14.1 (C-10') ppm. α=-9,3º

Decyl 2,6-dideoxy-β-L-*arabino*-hexopyranoside (12)

Using the above described protocol for the anomeric mixture (506 mg), gave **12** as a syrup (76 mg, 19%);

^{1}H NMR (400 MHz, CDCl3): δ = 4.49 (dd, 1H, H-1, $J_{1,2ax}$ = 9.73 Hz, $J_{1,2eq}$ = 1.89 Hz), 3.87 (ddd, 1H, H-1'a, $J_{1'a,2'}$ = 6.68 Hz, $J_{1'a,1'b}$ = 9.05 Hz), 3.66-3.57 (m, 1H, H-3), 3.28 (dq, 1H, H-5, $J_{5,4}$ = 8.83 Hz), 3.12 (t, 1H, H-4, $J_{4,3}$ = $J_{4,5}$ = 8.83 Hz), 2.22 (ddd, 1H, H-2eq, $J_{2eq,2ax}$ = 12.39 Hz, $J_{2eq,3}$ = 4.82 Hz), 1.68-1.55 (m, 3H, H-2ax, H-2'a, H-2'b), 1.47-1.20 (m, 17H, H-6, H-3'-H-9'), 0.89 (t, 3H, H-10', $J_{10',9'}$ = 6.84 Hz) ppm. 13C NMR (100 MHz, CDCl3): δ = 99.6 (C-1), 71.8 (C-4), 71.5 (C-5), 69.6 (C-1', C-5), 39.1 (C-2), 31.9 (C-1'), [29.6, 29.5, 29.4, 29.3, 26.0, 22.7] (C-3'-C-9'), 17.7 (C-6), 14.1 (C-10') ppm.

1H,1H Perfluorodecyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside (14)

Using the above described protocol for the anomeric mixture (1 g), gave **14** as a white powder (404 mg, 45%);

^{1}H NMR (400 MHz, CDCl3): δ = 4.85 (d, 1H, H-1, $J_{1,2ax}$ = 3.54 Hz), 4.00 (q, 1H, H-1'a, $J_{1'a,1'b} = J_{1'a,F2'a} = J_{1'a,F2'b}$ = 13.46 Hz), 3.86 (q, 1H, H-1'b, $J_{1'b,1'a} = J_{1'b,F2'a} = J_{1'b,F2'b}$ = 13.46 Hz), 3.75 (ddd, 1H, H-3), 3.59 (dq, 1H, H-5, $J_{5,4}$ = 9.47 Hz, $J_{5,6}$ = 6.19 Hz), 2.98 (t, 1H, H-4, $J_{4,3}$ = 9.22 Hz), 2.13 (ddd, 1H, H-2eq, $J_{2eq,1}$ = 1.01 Hz, $J_{2eq,3}$ = 5.18 Hz), 1.62 (ddd, 1H, H-2ax, $J_{2ax,2eq}$ = 13.19 Hz, $J_{2ax,3}$ = 11.62 Hz), 1.23 (d, 3H, H-6, $J_{6,5}$ =6,32 Hz) ppm. 13C NMR (100 MHz, CDCl3): δ = 98.4 (C-1), 77.2 (C-4), 68.5 (C-5), 68.1 (C-3), 63.8 (C-1'), 37.6 (C-2), 17.8 (C-6) ppm. Pf = 73,9 - 76,4 α= - 4,1º

1H,1H Perfluorodecyl 2,6-dideoxy-β-L-*arabino*-hexopyranoside (15)

Using the above described protocol for the anomeric mixture (1 g), gave **15** as a white powder (109 mg, 12%);

2-Octyldodecyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside (16)

Using the above described protocol for the anomeric mixture (1,2 g), gave **16** as syrup (518 mg, 51%);

^{1}H NMR (400 MHz, CDCl3): δ = 4.79 (br. s, 1H, H-1), 3.97-3.81 (m, 1H, H-3), 3.63 (dq, 1H, H-5, $J_{5,6}$ = 6.69 Hz), 3.52 (dd, 1H, H-1'a, $J_{1'a,1'b}$ = 8.46 Hz, $J_{1'a,2'}$ = 5.32 Hz), 3.21 (dd, 1H, H-1'b, $J_{1'b,2'}$ = 5.68 Hz), 3.10 (t, 1H, H-4, $J_{4,5} = J_{4,3}$ = 9.57 Hz), 2.12 (dd, 1H, H-2eq, $J_{2eq,2ax}$ = 12.63, $J_{2eq,3}$ = 4.80), 1.67 (br. t, 1H, H-2ax, $J_{2ax,3}$ =12,00), 1.56-1.48 (m, 1H, H-2'), 1.48-1.00 (m, 35H, H-6, H-3'- H-11', H-1''- H-8'') 1.00-0.76 (m, 6H, H-12', H-8'') ppm. 13C NMR (100 MHz, CDCl3): δ = 97.4 (C-1), 78.2 (C-4), 70.4 (C-1'), 69.4 (C-3), 67.5 (C-5), 38.0 (C-2'), 37.9 (C-2), [31.9, 31.4, 31.3, 30.9, 30.1, 30.0, 29.7, 29.6, 29.3, 26.9, 26.8, 26.7, 26.6] (C-3' to C-11' and C-1'' to C-7''), 17.7 (C-6), 14.1 (C-12', C-8'') ppm.

2-Octyldodecyl 2,6-dideoxy-β-L-*arabino*-hexopyranoside (12)

Using the above described protocol for the anomeric mixture (1,2 g), gave **12** as a syrup (186 mg, 18%);

9.1.4 General procedure for one-pot methodology

The nucleophile (4,668 mmol, 2 eq.) and a solution of TPHB (86,55 mg, 0.252 mmol) in dry CH_2Cl_2 (3.0 mL) (or CH_3CN for compounds **7,8**) were added to a solution of **3,4-diacetyl-6-deoxy-glucal** (500 mg, 2.334 mmol) in dry CH_2Cl_2 (3.0 mL). The mixture was stirred at room temperature for 50 min. After, CH_2Cl_2 (6 mL) was added to the reaction mixture and washed with a saturated solution of $NaHCO_3$ (or basic Amberlite is added and the solution is filtered, for compounds **7,8**). The solvent was evaporated, and the resulting residue was re-dissolved in MeOH (60 mL) and a solution of NaOMe in MeOH (1%, 10 mL) was added. The mixture was stirred at rt for 1 h. Neutralization with Amberlite (IR-120) was followed by filtration and evaporation of the solvent to give a residue, which was submitted to CC eluted with 1:1 EtOAc–n-hexane affording the corresponding 2,6-dideoxy glycosides.

Tridecyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside (6)

Using the above described protocol, gave **6** as a white powder (378 mg, 69%);

Tridecyl 2,6-dideoxy-β-L-*arabino*-hexopyranoside (7)

Using the above described protocol, gave 7 as a white powder (154 mg, 23%);

Dodecyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside (1)

Using the above described protocol, gave **1** as a white solid (497 mg, 67%);

Dodecyl 2,6-dideoxy-β-L-*arabino*-hexopyranoside (8)

Using the above described protocol, gave **8** as a white solid (108 mg, 14%);

Undecyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside (9)

Using the above described protocol, gave **9** as a white solid (418 mg, 56%);

^{1}H NMR (400 MHz, CDCl3): δ = 4.85 (d, 1H, H-1, $J_{1,2}$ = 2.94 Hz), 3.94 (ddd, 1H, H-3, $J_{3,2eq}$ = 5.05 Hz, $J_{3,2ax}$ = 12.41 Hz, $J_{3,4}$ = 9.19 Hz), 3.69-3.58 (m, 2H, H-5, H-1'a), 3.37 (dt, 1H, H-1'b, $J_{1'b,1'a}$ = 9.21 Hz, $J_{1'b,2}$ = 6.59 Hz), 3.12 (t, 1H, H-4, $J_{4,3}$ = $J_{4,5}$ = 9.09 Hz), 2.14 (dd, 1H, H-2eq, $J_{2eq,2ax}$ = 12.76 Hz), 1.69 (td, 1H, H-2ax, $J_{2ax,1}$ = 3.32 Hz), 1.60-1.51 (m, 2H, H-2'a, H-2'b), 1.40-1.14 (m, 17H, H-6, H-3'-H-9'), 0.81 (t, 3H, H-10', $J_{10',9'}$ = 7.04 Hz) ppm. 13C NMR (100 MHz, CDCl3): δ = 97.2 (C-1), 78.1 (C-4), 69.3 (C-5), 67.4 (C-1'), 67.3 (C-5), 37.9 (C-2), 31.9 (C-1'), [29.6, 29.5, 29.4, 29.3, 26.2, 22.7] (C-3'-C-9'), 17.7 (C-6), 14.1 (C-10') ppm.

Undecyl 2,6-dideoxy-β-L-*arabino*-hexopyranoside (10)

Using the above described protocol, gave **10** as a white solid (107 mg, 15%);

^{1}H NMR (400 MHz, CDCl3): δ = 4.48 (d, 1H, H-1, $J_{1,2}$ = 9.66 Hz), 3.87 (ddd, 1H, H-3, $J_{3,2eq}$ = 5.05 Hz, $J_{3,2ax}$ = 12.10 Hz, $J_{3,4}$ = 9.09 Hz), 3.61-3.49 (m, 2H, H-5, H-1'a), 3.27 (dt, 1H, H-1'b, $J_{1'b,1'a}$ = 9.41 Hz, $J_{1'b,2}$ = 6.59 Hz), 3.03 (t, 1H, H-4, $J_{4,3}$ = $J_{4,5}$ = 9.09 Hz), 2.05 (dd, 1H, H-2eq, $J_{2eq,2ax}$ = 12.76 Hz), 1.60 (td, 1H, H-2ax, $J_{2ax,1}$ = 3.50 Hz), 1.54-1.43 (m, 2H, H-2'a, H-2'b), 1.40-1.14 (m, 17H, H-6, H-3'-H-9'), 0.81 (t, 3H, H-10', $J_{10',9'}$ = 7.04 Hz) ppm. 13C NMR (100 MHz, CDCl3): δ = 97.2 (C-1), 78.1 (C-4), 69.3 (C-5), 67.4 (C-1'), 67.3 (C-5), 37.9 (C-2), 31.9 (C-1'), [29.6, 29.5, 29.4, 29.3, 26.2, 22.7] (C-3'-C-9'), 17.7 (C-6), 14.1 (C-10') ppm.

Decyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside (11)

Using the above described protocol and **3,4-diacetyl-6-deoxy-glucal** (400 mg, 1.867 mmol), gave **11** as syrup (384 mg, 71%);

Decyl 2,6-dideoxy-β-L-*arabino*-hexopyranoside (12)

Using the above described protocol and **3,4-diacetyl-6-deoxy-glucal** (400 mg, 1.867 mmol), gave **12** as a syrup (79 mg, 15%);

1H,1H Perfluorodecyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside (14)

Using the above described protocol, gave **14** as a white powder (863 mg, 59%);

1H,1H Perfluorodecyl 2,6-dideoxy-β-L-*arabino*-hexopyranoside (15)

Using the above described protocol, gave 15 as a white powder (250 mg, 17%);

9.1.5 General procedure for the microwave assisted glycosylation and one-pot deacetylation.

A solution of nucleophile (4,668 mmol, 2 eq.) and TPHB (86,55 mg, 0.252 mmol) in dry CH_2Cl_2 (3.0 mL) were added to a solution of **3,4-diacetyl-6-deoxy-glucal** (500 mg, 2.334 mmol) in dry CH_2Cl_2 (3.0 mL). The mixture was stirred and irradiated with micro-waves (400nm) room temperature for 50 min. After, CH_2Cl_2 (6 mL) was added to the reaction mixture and washed with a saturated solution of $NaHCO_3$ (or basic Amberlite is added and the solution is filtered, for compounds **14,15**). The solvent was evaporated, and the resulting residue was re-dissolved in MeOH (60 mL) and a solution of NaOMe in MeOH (1%, 10 mL) was added. The mixture was stirred at rt for 1 h. Neutralization with Amberlite (IR-120) was followed by filtration and evaporation of the solvent to give a residue, which was submitted to CC eluted with 1:1 EtOAc–n-hexane affording the corresponding 2,6-dideoxy glycosides.

Dodecyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside (1)

Using the above described protocol, gave 1 as a white solid (63% yield);

Dodecyl 2,6-dideoxy-β-L-*arabino*-hexopyranoside (8)

Using the above described protocol, gave 8 as a white solid (24% yield);

Decyl 2,6-dideoxy-α-L-*arabino*-hexopyranoside (11)

Using the above described protocol and **3,4-diacetyl-6-deoxy-glucal** (200 mg, 0.934 mmol), gave 11 as syrup (152 mg,56%);

Decyl 2,6-dideoxy-β-L-*arabino*-hexopyranoside (12)

Using the above described protocol and **3,4-diacetyl-6-deoxy-glucal** (200 mg, 0.934 mmol), gave 12 as a syrup (48 mg, 18%);

9.2 Synthesis of Alzheimer's therapeutics

9.2.1 General procedures for glucosyl donor synthesis

9.2.1.1 Synthesis of D-glucose pentaacetate

To a solution of the commercial free glucose (6 mmol) in pyridine (10 mL/g), acetic anhydride (2 eq./OH, 4.5 mL, 48 mmol) was slowly added and, finally, a catalytic amount of DMAP (ca. 20 mg). Reaction mixture was left to stir at room temperature for two hours. Then, the solution was diluted with dichloromethane (30 mL) and washed 3 times with HCl 2M and finally with brine. The organic phase was dried with anhydrous $MgSO_4$, filtered and concentrated under vacuum, affording the peracetylated sugar as a colourless oil. This reaction provided quantitative yields of pure products, as verified by TLC and NMR, without further purification being necessary.

9.2.1.2 Synthesis of D-glucose bromide

Method 1: The peracetylated glycoside was then dissolved in glacial acetic acid (5 mL/g) to which acetic anhydride (1.5 eq.) and hydrobromic acid (33%) (3 eq.) were added. Reaction mixture was kept stirring at room temperature protected from light and under nitrogen. After 3h, reaction mixture was diluted with dichloromethane and washed 3 times with saturated $NaHCO_3$ and with brine. The organic phase was dried with anhydrous $MgSO_4$, filtered and concentrated under vacuum, affording the corresponding glycosyl bromide in low yields (Table 20) as a translucent yellow oil. Alternatively, modifications in the solvent mixture were performed in an attempt to optimize the results: **Method 2**: The peracetylated glycoside was dissolved in dichloromethane, and acetic anhydride and hydrobromic acid (33%) were added. **Method 3**: The peracetylated glycoside was dissolved in hydrobromic acid (33%).

Method 4: The resulting product was then dissolved in glacial acetic acid (5 mL/g) to which methanol (1.5 eq.) and acetyl bromide (3 eq.) were added. Reaction mixture was kept stirring at room temperature protected from light and under nitrogen. After 3h, reaction mixture was diluted with dichloromethane and washed 3 times with saturated $NaHCO_3$ and with brine. Organic phase was dried with anhydrous $MgSO_4$,

filtered and concentrated under vacuum, affording the corresponding glycosyl bromide as a translucent yellow oil.

9.2.2 Procedures for the glycoconjugation reaction and deprotection

9.2.2.1 Synthesis using D-glucose pentaacetate as a glycosyl donor

Method 1.1: In a flask, make a solution of per-acetylated glucose (2 eq) and the aglycone dissolved in DCM (5 mL/g). Then add trimethylamine (1,1 eq) dissolved in DCM and successively boron trifluoride etherate (2,5 eq).

Method 1.2: In a flask, make a solution of per-acetylated glucose (1,1 eq) and the aglycone dissolved in DCM (5 mL/g). Then add trimethylamine (0,5 eq) dissolved in DCM and then add also boron trifluoride etherate (2,5 eq)

Method 1.3: In a flask, make a solution of per-acetylated glucose (5 eq) and the aglycone dissolved in DCM (5 mL/g). Then boron trifluoride etherate (2,5 eq) was added.

The reaction mixture was protected from light and placed under agitation at room temperature. It was controlled by TLC and after completion it was added water to the reaction, and neutralized, and then extracted with ethyl acetate.

9.2.2.2 Synthesis using D-glucose bromide as a glycosyl donor

A solution of glucobromide (2 eq), the aglycone and the phase-transfer catalyst in DCM (10 mL/g) was protected from light and placed under agitation at room temperature. After, a similar volume of an aqueous solution of the desired base was added. When it is complete, dilute with DCM, neutralize the solution, and wash with water. The organic phase is then dried with CaSO4 and evaporated. After purification by chromatographic column (Ethyl acetate: Hexane), the corresponding glucoconjugate is obtained as a white solid.

9.2.2.3 General procedure for the deacetylation reaction

A solution of NaOMe in MeOH (1%, 10 mL) was added to a solution of the acetylated sugar (1.3 mmol) in MeOH (60 mL) and the mixture was stirred at rt for 1 h. Neutralization with Amberlite (IR-120) was followed by filtration and evaporation of the solvent, and the resulting residue was submitted to purification by chromatographic

column, eluted with 1:1 EtOAc–n-hexane affording the corresponding 2,6-dideoxy glycosides.

Dissolve the acetylated sugar in methanol (100 mL/g) and place under agitation. Then add drop by drop a previously prepared solution of 10% NaOMe in MeOH (10 mL/g). After the reaction is complete, neutralize the solution, and evaporate the solvent.

For this case, the neutralization was made with HCl solution, which was problematic once the product was highly soluble in water. The water was evaporated, and the compound dissolved in ethanol, filtered and the product was then obtained pure.

9.3 Biological assays

9.3.1 Bacterial Cultures

Strains of *B. cereus* ATCC 11778, *B. cereus* ATCC 14579, *B. subtilis* ATCC 6633, *E. coli* ATCC 8739 and *S. aureus* ATCC 29213 were obtained from the American Type Culture Collection (Rockville, MD). The bacterial cultures were maintained at lyophilized state or stored at -80ºC in Muller-Hinton broth (MH) supplemented with 20% glycerol (Sigma Aldrich). Overnight cultures of bacteria were grown in 50 mL Erlenmeyer flasks, with orbital shake at 200 rpm at 30 ºC.

9.3.2 MIC and MBC determination

MIC determination was made by a modified micro-dilution method according to CLSI guidelines[214]. Briefly, overnight culture (17 h) of all bacterial strains were diluted to 0,5 McFarland units in Muller-Hinton broth. In sterile 96-well plates (Sarstedt, Germary), 150 µL of culture media were added and then supplemented with the compound in study by serial dilution. Finally, the desired wells were inoculated with 10 µL of a 1/10 dilution of the 0,5 McFarland bacterial suspension. Microplates were incubated at 35 °C, with orbital shaking, taking absorbance readings is all wells at intervals of 10 minutes, for up to 24 h (Anthos Zenith 3100 Microplate Multimode Detector). The lowest concentration at which no bacterial growth was observed is considered the minimal inhibitory concentration. Aliquots of 5 µL from every well were transferred to Muller-Hinton Agar (MHA) for determination of minimum bactericidal concentration (MBC). Bacterial growth was assessed at 17 h, MBC was considered the lowest concentration of compound where no bacterial growth was observed. All measurements are the average of a minimum of 3 independent experiments.

9.3.3 Cytotoxicity evaluation in Caco-2 cells

9.3.3.1 Cell cultures

Caco-2 cells were grown in DMEM high glucose supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, in a humidified incubator at 37 °C with a 5 % CO2 atmosphere. The cells were trypsinized twice a week with trypsin/EDTA (0.05%/0.02%) and the medium was also changed twice a week.

9.3.3.2 Cell viability assay

Determination of cell growth was performed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. On day 1, 20,000 cells/well were seeded into 96-well plates in a volume of 100 µL. On day 2, the different drugs concentration (0.1–100 µM) were added to the plate. In all the experiments, the various drug-solvents (EtOH, DMSO) were added in each control to evaluate a possible solvent cytotoxicity. After the established incubation time with drugs, MTT (0.5 mg/mL) was added to each well, and after 3-4 h incubation at 37 °C, the supernatant was removed. The formazan crystals were solubilized using 100 µL of DMSO/EtOH (1:1) and the absorbance values at 570 nm and 630 nm were determined on the microplate reader Victor3 from Perkin Elmer Life Sciences.

9.3.4 In vitro Time-Killing curves:

Inoculum preparation: The initial cellular concentration was standardized in MH by OD_{600nm} in order to obtain 10^8, 10^7 and 10^6 cells per microplate well, as needed.

Microplate preparation: Stock solution of compound **1** dissolved in DMSO (2.4 µl) was serial diluted (2-0,25X MIC) in 150 µl of sterile MH. The plate inoculation is performed by adding 10 µl of the inoculum previously prepared.

Time-kill assay conditions: All the microplates prepared were incubated at 35ºC, with orbital shaking at 200rpm, the OD readings were performed at 600 nm (Anthos Zenith 3100 Microplate Multimode Detector). After each OD reading, the plate is retrieved, and 5 µl are taken and added to a premade plate containing 95 µl of sterile medium followed by 7 steps of 1/10 serial dilution.

Colony Forming Units (CFU) counting: After the serial dilution step, a 10 µl drop from every well is placed in MHA solid media plates. After inoculation, the plates are incubated for 17 h at 30ºC for later CFU counting.

The compounds were added to an exponential phase culture of *B. cereus* in Muller-Hinton containing about 10^6, 10^7, and 10^8 cells/mL. Bacteria were then incubated at 35 ºC, and 5 µL aliquots were withdrawn at different intervals and diluted in a previously premade microplate. The samples were serial diluted in 1/10 of the previous concentration, and then, a 10 µL droplet was transferred to MHA and bacterial growth

was evaluated. The number of surviving bacteria, expressed as the number of colony forming units (CFU), were counted after an overnight incubation at 30 ºC.

For all the experiments, sample homogeneity and methodology validation was performed by OD_{600nm}, as a measure of cellular density, for all sixteen replicates.

9.3.5 Compound 1 bioactivity against bacterial strains resistant to conventional antibiotics

Resistant mutants to the major families of antibiotics were created. This was accomplished using a variant of the gradient plate methodology for the following antibiotics of diverse classes: erythromycin (macrolide), penicillin, vancomycin (glycopeptide), ciprofloxacin (quinolone), tetracycline (Sigma Aldrich). The plates were inoculated with 100 µL of 0.5 McFarland bacterial suspension of *B. cereus* ATCC 14579 strain, carefully dispersed on the agar surface using spreading spheres. The plates were incubated at 30 ºC during 24 hours. After, the colony closest to the higher concentration in the gradient was collected, re-suspended and re-inoculated in new fresh gradient plates. This procedure was repeated for 15 days.

9.3.6 Susceptibility of *Bacillus* spores to compound 1

Production of spores from *B. cereus* ATCC 14579 was carried out by incubation at 37 ºC in sporulation medium (CCY – *Bacillus* Sporulation media[217]: 1g/L acid casein hydrolysate, 1 g/L bacto casitone (pancreatic casein hydrolysate), 0.4 g/L yeast extract, 20 mg/L L-glutamine, 0.06% (v/v) glycerol, 1.77 g/L KH_2PO_4, 4.53 g/L K_2HPO_4, 0.5mM $MgCl_2.6H_2O$, 0.01mM $MnCl_2.4H_2O$, 0.05 mM $ZnCl_2$, 0.2 mM $CaCl_2.6H_2O$, 0.05 mM $FeCl_3.6H_20$.) for 96 hours. Purification of the spores was achieved by centrifugation at 4 ºC, 15000 g and wash 10 times with Milli RO water. As preparation for the susceptibility assays, the spores were suspended in 1 mM PBS and 0,01 % tween 20, then submitted to heat treatment at 70 ºC for 30 minutes. Germination was achieved in AGFK buffer (30 mM L-asparagine, 5,6 mM D-Glucose, 5,6 mM D-Fructose, 20 mM KCl and 50 mM Tris-HCl, pH 8,4) at 37 ºC. Susceptibility to compound **1** was assessed at all the preparation steps, 20 minutes and 2 hours after the germination process.

9.3.7 Differential metabolomics analysis

The study of bacterial metabolic response to compound **1**, was performed using Phenotype MicroArrays (Biolog). The impact of compound **1** was assessed in the metabolism of 95 different carbon sources by *B. cereus* ATCC 14579 strain. All the solutions used were supplied by the manufacturer. The PM solutions B-F were sterilized in autoclave at 121 ºC during 15 minutes, and afterwards stored at 4 ºC until use. The PM additive solution was prepared by mixing these solutions (10 mL of solutions B, C, E and F and 30 mL of solution D and water). The bacterial growth media was used according to instructions provided by the manufacturer. The bacterial suspension, for PM Inoculating Fluid, was prepared by centrifuging the overnight pre-inoculum (at 3000g, 4 ºC for 5 minutes) collecting and resuspending the cells in IF-0a, and finally adjusting the bacterial suspension to 0.5 McFarland Units.

Finally, 100 μL of the PM inoculating fluid, was added to each well of 4 PM1 microplates (Compound **1** at the final concentrations of 16, 8, 4 and 0 μg/mL). The microplates were sealed with parafilm, shielded from light and incubated at 35 ºC with orbital shaking. The compound was added at 1 hour of incubation, in order to stabilize and allow bacterial cells to adapt to the new media.

The reduction of tetrazolim violet associated to NADH produced by the consumption of each tested carbon source, was monitored by OD_{590nm} at time 0', at 30', at 60' after incubation, and then, hourly for 27 hours. (Anthos Zenith 3100 Microplate Multimode Detector).

9.3.7.1 Data Analysis

The time-dependent series for each tested carbon source was classified by the acquisition periods, corresponding to: 1) initial phase of response to the compound **1** (from 0 to 5 hours of incubation); 2) late phase of response (from 16 to 27 hours of incubation).

After data normalization, results were divided into five categories depending on the effect shown: 1) highly inhibitory effect, when the difference between the effect of the compound **1** and control was higher than 5σ ($OD_{590nm}>5\sigma$); 2) inhibitory effect, when the difference observed was between 5σ and 2σ ($5\sigma>OD_{590nm}>2\sigma$); 3) no effect, when bacterial growth was observed in the absence of compound ($OD_{590nm}>2\sigma$) and the

difference between absence and presence of compound was less than 2σ; 4) no growth, when value with and without the presence of compound was less than 2σ (OD_{590nm}<2σ); 5) potentiation effect, when value without the compound was less than 2σ (OD_{590nm}<2σ) and the difference of value between the presence and absence of compound was superior to σ.

The hits classified as category 1, 2 or 5, were correlated to the biological pathways described at "Kyoto Encyclopedia of Genes and Genomes" (KEGG) database (*33*). The biological pathways were then ordered by the number of inhibitory hits per pathway. The metabolic reconstruction was performed using graph theory for hierarchical superimposition of the pathways based at the nonparametric Spearman's rank association coefficients (edges) between hits (nodes – according to KO classification system). The SPSS (SPSS Statistics for Windows, SPSS Inc.) software was used for data normatization and determination of nonparametric association coefficients and Cytoscape (Cytoscape, U.S. NIGMS) for the hierarquical representation (Fig. S3).

9.3.8 Mutant libraries

9.3.8.1 Preparation of electro-competent cells

B. cereus ATCC 14579 cells were grown in a Erlenmeyer flask containing tryptic soy broth (TSB, Difco Laboratories), and placed in an incubator at 37ºC and 200 rpm, until the bacterial suspension reaches a maximum OD of 0.45 – 0.46 measured at 600 nm. The suspension was then incubated during 1 h in 5% glycine or DL-threonine and 250 mM sucrose at 37 °C at 200 rpm. Cells were washed five times in electroporation buffer (250 mM sucrose, 1 mM Hepes, 1 mM MgCl2, 10% glycerol, pH 7.0) and concentrated 150-fold.

9.3.8.2 Electroporation

Electroporation was performed at 25 μF using a Bio-Rad Gene Pulser X-cell apparatus (Bio-Rad laboratories, U.S.). Electroporation was carried out in 1 mm electroporation cuvettes, at 4ºC, with 50 μl cells combined with 500 ng of plasmid, in order to respect cells/DNA proportions. It was used voltage of 20kV/cm^{-1} with 200 ohms of resistance. After electroporation, cell suspensions were immediately diluted with 1 ml of TSB supplemented with 250 mM sucrose, 5 mM MgCl2, 5 mM MgSO4 and incubated for 2 h at 37 °C, 200 rpm to allow expression of antibiotic resistance markers. Aliquots

were spread onto tryptic soy agar (TSA, Difco Laboratories) supplemented with appropriate antibiotic. Transformants harbouring antibiotic resistance were counted following overnight incubation.

9.3.8.3 Construction of in vivo mutant library by random insertion of transposon Tn917 in B. cereus *ATCC 14579*

Transposon mutagenesis was performed by obtaining a stationary-phase culture of modified *B. cereus* (pLTV1) cultured overnight at 28°C in Lysogeny broth (LB) containing tetracycline (50 µg/ml), erythromycin (1 µg/ml), and chloramphenicol (10 µg/ml) (Sigma Aldrich). This culture was diluted 1:800 in prewarmed (43°C) LB broth that contained erythromycin (1 µg/ml) and chloramphenicol (5 µg/ml). Then, it was incubated at 43°C for 24 h with shaking at 200 rpm. The above procedure was repeated one more time. Transposon mutants were subsequently selected on LB agar containing erythromycin (1 µg/ml) and chloramphenicol (5 µg/ml) and incubated at 37°C, resulting in two libraries of 10^6 mutants each.

9.3.8.4 Southern Blotting

The validation of insertion site of Tn917 carried by plasmid pLVT1 was performed in the two pool of mutants. Briefly, chromosomal DNA from both *B. cereus* ATCC 14579 wild and modified strains, as well as from both generated mutant libraries, were isolated using the CTAB method (*34*). After digestion with *Eco*RI the DNA of the mutant libraries and control strains were resolved in an agarose gel 0.8 %. The DNA transfer proceeded overnight and hybridization was performed at 40 ºC with DNA probe (5.1 kb *Eco*RI restriction fragment of Tn917), labelled with digoxigenin-dUTP, at a concentration of 25 ng/mL. The membrane was revealed with NBT-BCIP.

9.3.8.5 Transporter systems knockout mutant library

Twenty-eight knockout mutants based in the isogenic system *B. subtilis* 168 on main ABC and PTS systems were purchased from National BioResource Project (NIG, Japan): *Bacillus subtilis*. Table 18 identifies the individual mutants and respective MIC and MBC values obtained for compound **1**.

9.3.9 Susceptibility of bacterial protoplasts and spheroplasts to compound 1

In order to understand the role of the ultrastructure of cellular envelope, the susceptibility of Gram-negative *E. coli* K12 spheroplasts, Gram positive *B. cereus* ATCC 14579 and *S. aureus* ATCC 29213 protoplasts were evaluated. Bacterial spheroplasts and protoplasts [LPS and peptidoglycan-free bacteria] were prepared by harvesting bacterial cells by centrifugation (10, 000 g during 10 minutes at 4 ºC), and washing with 10 mM Tris-HCl (pH 8) at 4 ºC. Cells were resuspended, supplemented with sucrose (0.6 M) at room temperature. Lysozyme (0.5 mg/mL) (Sigma Aldrich) was added, very slowly aiming to decrease the aggregation of plasts, after 30 min of incubation. The plasts (used at the same concentration as in the biological activity assay) were incubated with the desired compound and with polymixin B as a positive control, at various concentrations.

9.3.10 Morphologic evaluation by atomic force microscopy

Samples containing *B. cereus* ATCC 14579 (10^6 CFU/mL) in MH medium were incubated with compound **1** at various concentrations (0, 8 and 16 µg/mL) and after 2 hours aliquots were taken. A drop (40 µL) containing the bacteria was deposited onto freshly cleaved mica surfaces for 30 minutes, gently washed with Milli-Q water and dried under mild nitrogen flux. The surface was examined ex-situ, at ambient temperature (21ºC), using Nanoscope IIIa multimode atomic force microscope (Digital Instruments, Veeco, Santa Barbara, CA) and etched silicon tips (TESP-V2, Bruker) with a resonance frequency of ca. 300 kHz. Images were acquired with scan rates between 1.2 and 1.5 Hz. It is worth to note that washing and drying steps, as wells as tip repetitive scanning did not influence the results presented in this work.

9.3.11 Methodologies used in surface disinfection assays

For all methodological variations, the bacterial strain in study was *B. cereus* ATCC 14579, the liquid growth media used was Muller-Hinton (MH) and the solid growth media was Muller-Hinton Agar (MHA). Concentrations tested for compound 1 were 8 mg/L, 16 mg/L, 32 mg/L, 256 mg/L and 1000 mg/L.

Method 1: according to NP1040 from OECD "Guidelines for the Testing of Chemicals" - "Quantitative Method for Evaluating Bactericidal Efficacy of Biocides Used on Hard Surfaces", a 10 µL drop of standardized bacterial suspension was placed in a

sterile surface and allowed to dry. After, a 15-20 μL drop of compound 1 solution in water (at the desired concentration) was added to the surface, in such a way that completely covers the inoculated surface. After the desired time period, the surface is washed with growth media as means of neutralizing the compound in action. The media used in the wash was recovered and aliquots were plated in solid growth media (MHA) and further incubated. Results obtained by observation at 24 hours proved to be inconsistent and of low reproducibility. For this reason, results presented were obtained by growth observation at 48 hours.

*results defined as not determined (nd) were inconclusive results for lack of reproducibility.

Method 2: A variation to the standard methodology was deemed necessary since, while tuning the method, it was concluded that the surface washing and sampling steps lead to low reproducible results. So, instead, the smaller surfaces, after the desired time for the action of compound 1, were submerged in liquid growth media and vortexed. Then, samples of this media were plated in solid growth media, and incubated for 48h.

Method 3: Since method 2 could not be applied to all surfaces, the assessment of the efficacy of compound 1 was performed by direct transfer: the surfaces were placed in contact with solid growth media and removed after a short period of time, and the media was incubated for 48h.

Method 4: As a more practical alternative to method 3, after the desired time period for the compounds action, a swab sampling was performed and the swab was placed on solid growth media and dampened with sterile media if needed for complete adhesion. Alternatively, the swabs were also immersed in liquid growth media. Both methodologies provided similar results after 48h incubation period.

Method 5: since the obtained results were not expected, the dry, contaminated surfaces were completely submerged in growth media supplemented with compound 1, as an attempt at approximate the conditions to the ones known to be optimal for the compounds action. After submersion, the results were obtained by direct transfer methodology.

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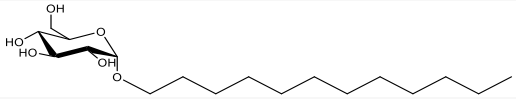
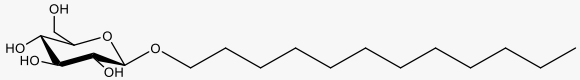
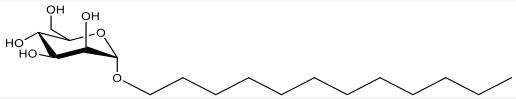
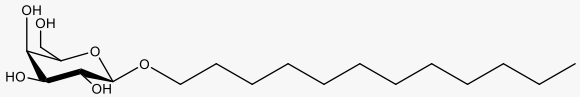
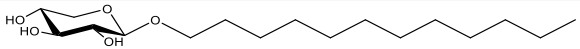
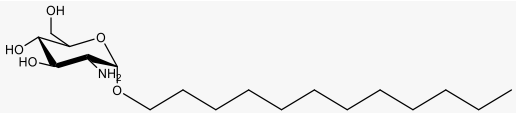
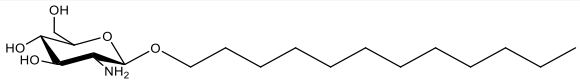
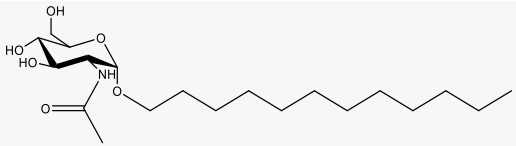
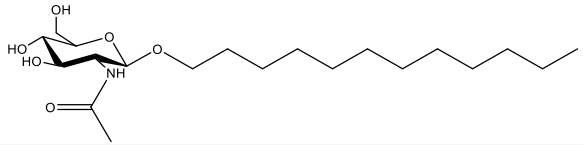
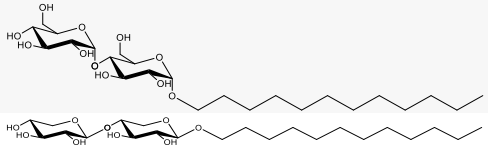
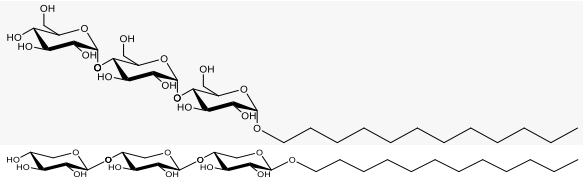
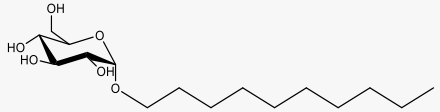
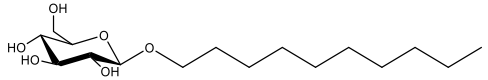
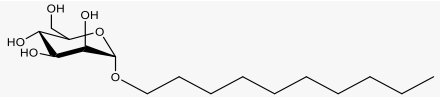
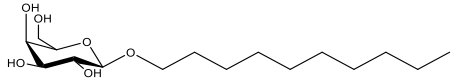
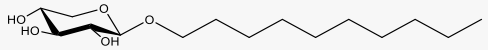
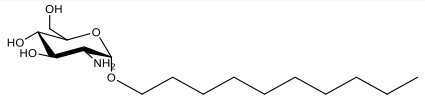
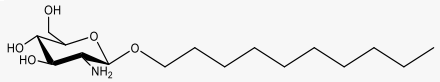
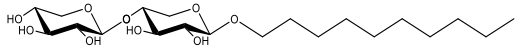
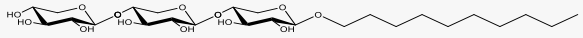
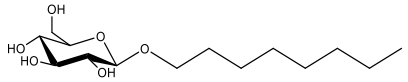
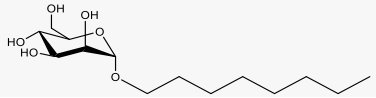
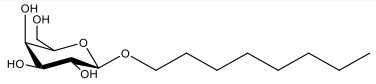
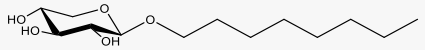
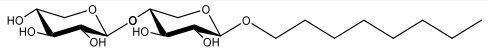
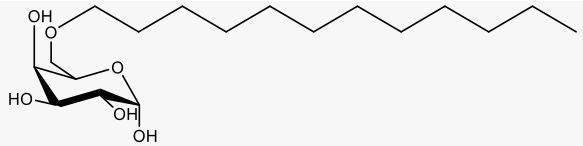
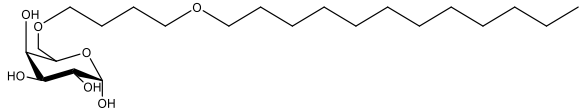
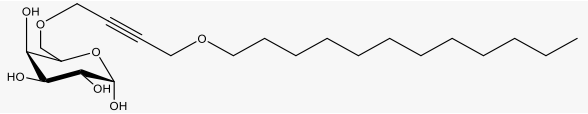
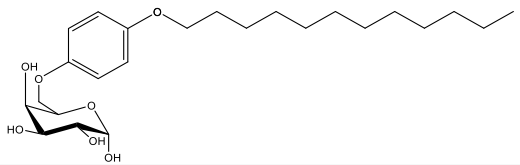
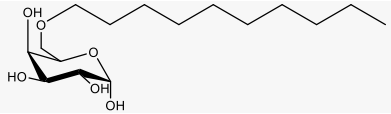
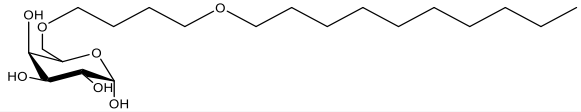
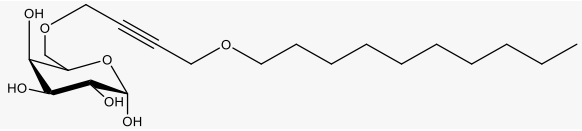
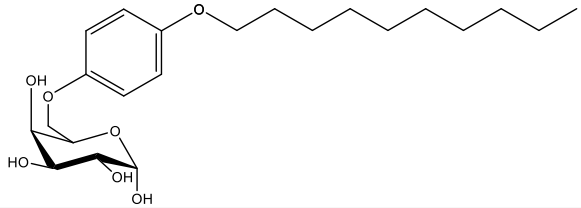
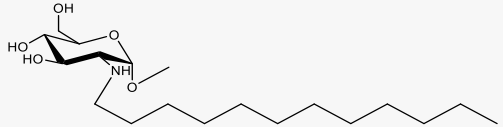
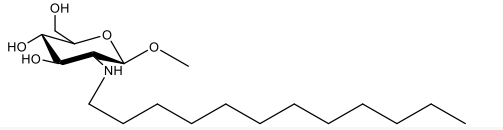
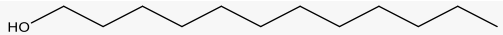
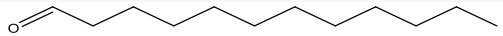
Annex

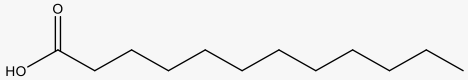
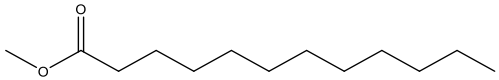
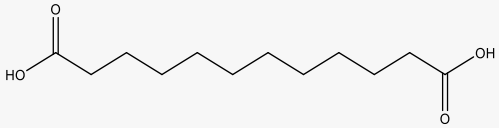
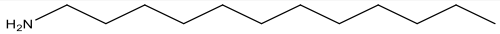
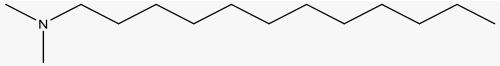
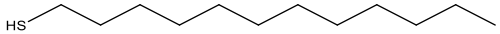
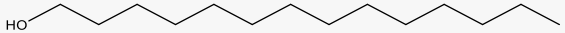
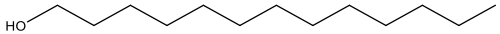
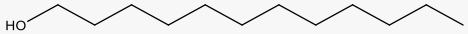
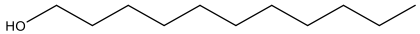
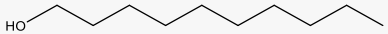
Annex 1. Summary of similar compounds found in the literature, as well as similar aglycones, and their biological activity in the most relevant microorganisms, for comparison (MIC [mM])

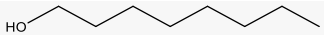
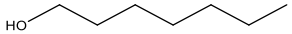
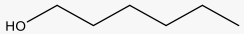
Structure	*S. aureus*	*B. subtilis*	*E. coli*	*P. aeruginosa*	*C. albicans*	*S. cerevisiae*	*A. Niger*	*P. chrysogenum*
	0,029	0,072	0,574	1,148	1,148	1,148	0,574	0,574
	0,072	0,143	>1,148	>1,148	0,143	0,072	0,574	0,574
	0,072	0,072	>1,148	>1,148	0,574	0,029	1,148	0,574
	0,574	0,574	>1,148	>1,148	>1,148	0,574	>1,148	>1,148
	1,256	0,628	>1,256	>1,256	>1,256		1,256	1,256
	0,029	0,029	0,144	0,029	0,576	0,029	1,151	0,576
	0,029	0,029	0,144	0,072	1,151	0,072	1,151	0,576
	>1,027	>1,027	>1,027	>1,027	>1,027	>1,027	>1,027	>1,027

	>1,027	>1,027	1,027	>1,027	>1,027	>1,027	>1,027	1,027
	>1,95	>1,95	>1,95	>15,6	0,243		0,122[1]	
	0,111	0,111	0,888	0,888	0,222		0,222	0,222
	0,089	0,178	0,178	>1,42	0,355		0,355[1]	
	>0,686	0,686	>0,686	>0,686	>0,686		>0,686	0,686
	0,312	0,312	0,624	0,624	>1,248	0,312	>1,248	>1,248
	0,312	0,312	0,624	0,624	0,624	0,312	1,248	1,248
	0,156	0,156	0,624	0,624	0,624	0,156	>1,248	1,248
	0,312	0,312	1,248	1,248	0,624	0,312	1,248	0,624
	>1,377	>1,377	>1,377	>1,377	>1,377		>1,377	1,377
	0,313	0,313	0,626	0,157	1,252	0,313	1,252	1,252

	0,157	0,313	0,626	0,157	1,252	0,313	>1,252	1,252
	0,473	0,473	>0,947	>0,947	>0,947		0,947	0,947
	>0,721	>0,721	>0,721	>0,721	>0,721		>0,721	>0,721
	1,368	1,368	1,368	1,368	>1,368	>1,368	>1,368	1,368
	0,342	>1,368	>1,368	1,368	1,368	1,368	>1,368	1,368
	0,684	1,368	1,368	1,368	>1,368	>1,368	>1,368	1,368
	>1,525	>1,525	>1,525	>1,525	>1,525		>1,525	>1,525
	>1,014	>1,014	>1,014	>1,014	>1,014		>1,014	>1,014
	0,717	0,717[2]	0,717	0,717				
	0,297	0,074[2]	0,149	0,149				
	0,150	0,019[2]	0,150	0,150				

	0,274	$0,137^2$	0,137	0,137			
	0,780	$0,780^2$	0,780	0,780			
	0,318	$0,159^2$	0,159	0,159			
	0,322	$0,161^2$	0,161	0,322			
	0,292	$0,146^2$	0,146	0,292			
	0,277	0,277	0,014	0,028	0,553	0,138	>1,106	0,138
	0,138	0,138	0,069	0,028	0,277	0,138	1,106	0,138
	0,271				0,135			
	0,272				0,135			

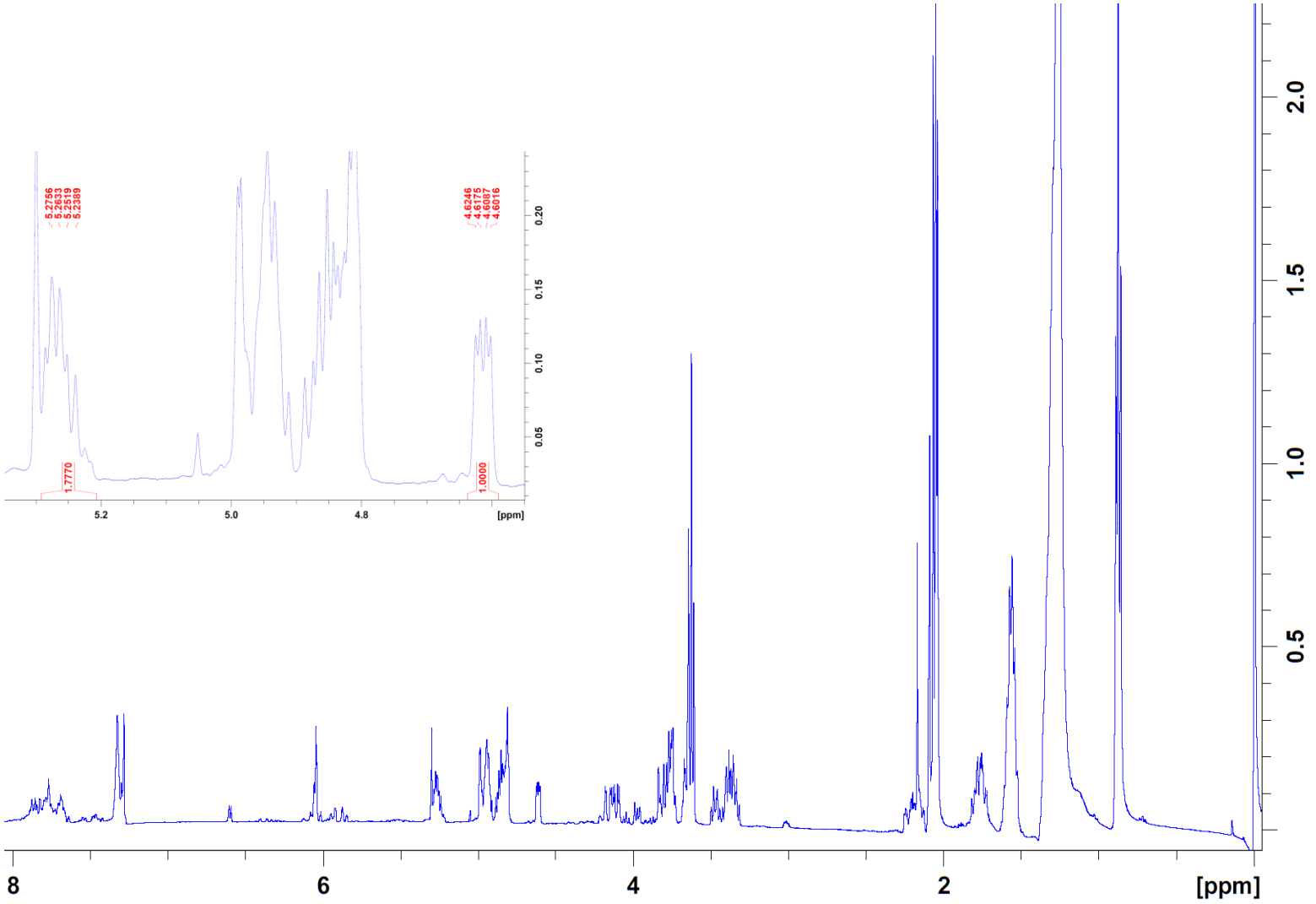
	2,490				2,490			
	>4,6				4,6			
	>4,3				>4,3			
	0,112				0,112			
	0,109				0,109			
	>4,94				>4,94			
	>3,732	>3,732	>3,732	>3,732		>3,732	>3,732	>3,732
	>3,993	0,031	>3,993	>3,993		>3,993	>3,993	3,993
	0,067	0,067	>4,293	>4,293		>4,293	>4,293	0,067
	0,145	0,145	>4,643	>4,643		0,290	0,145	0,073
	0,316	0,316	>5,054	>5,054		0,316	0,316	0,158
	1,386	1,386	>5,546	>5,546		0,693	1,386	0,347

	6,143	3,072	>6,143	3,072	3,072	3,072	1,536
	>6,885	>6,885	>6,885	6,885	6,885	6,885	3,442
	>7,830	>7,830	>7,830	>7,830	>7,830	>7,830	7,830

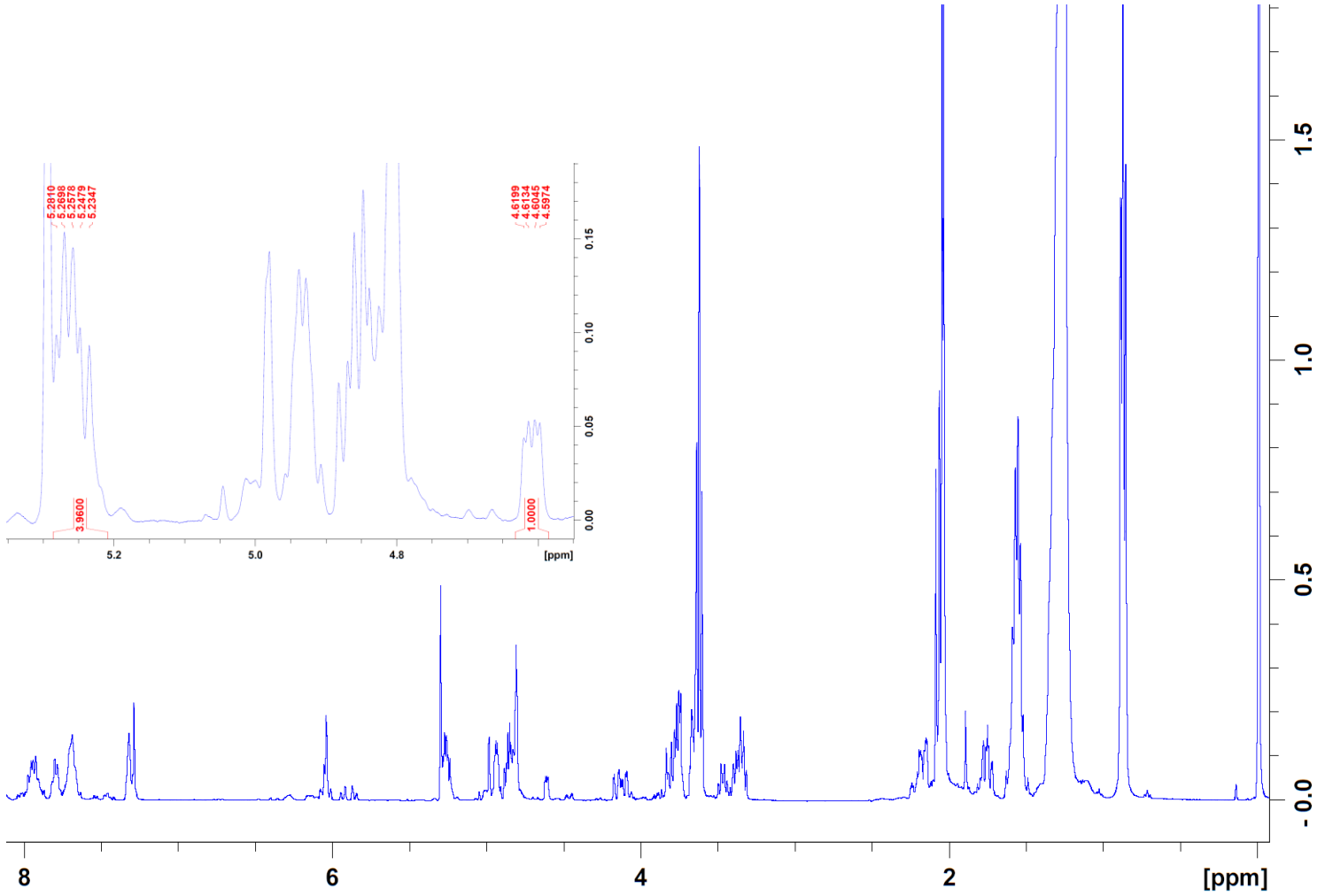
[1] Different fungal strain: *Aspergillus brasiliensis*
[2] Different bacterial strain: *Bacillus stearothermophilus*

Annex 2. NMR comparison of reactional mixtures and corresponding α:β ratios.

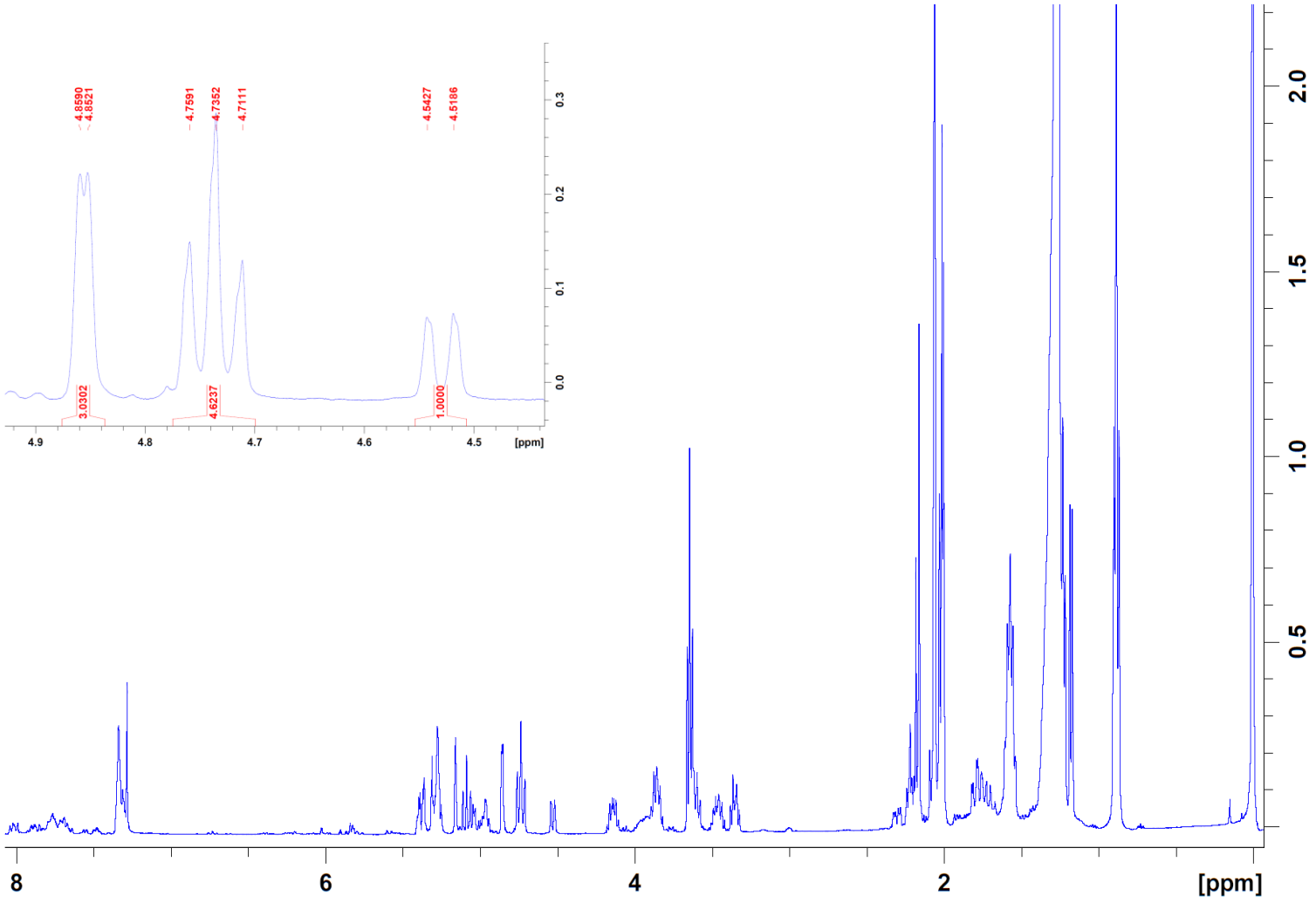
Annex 2. A - NMR from reactional system a, in CH_2Cl_2



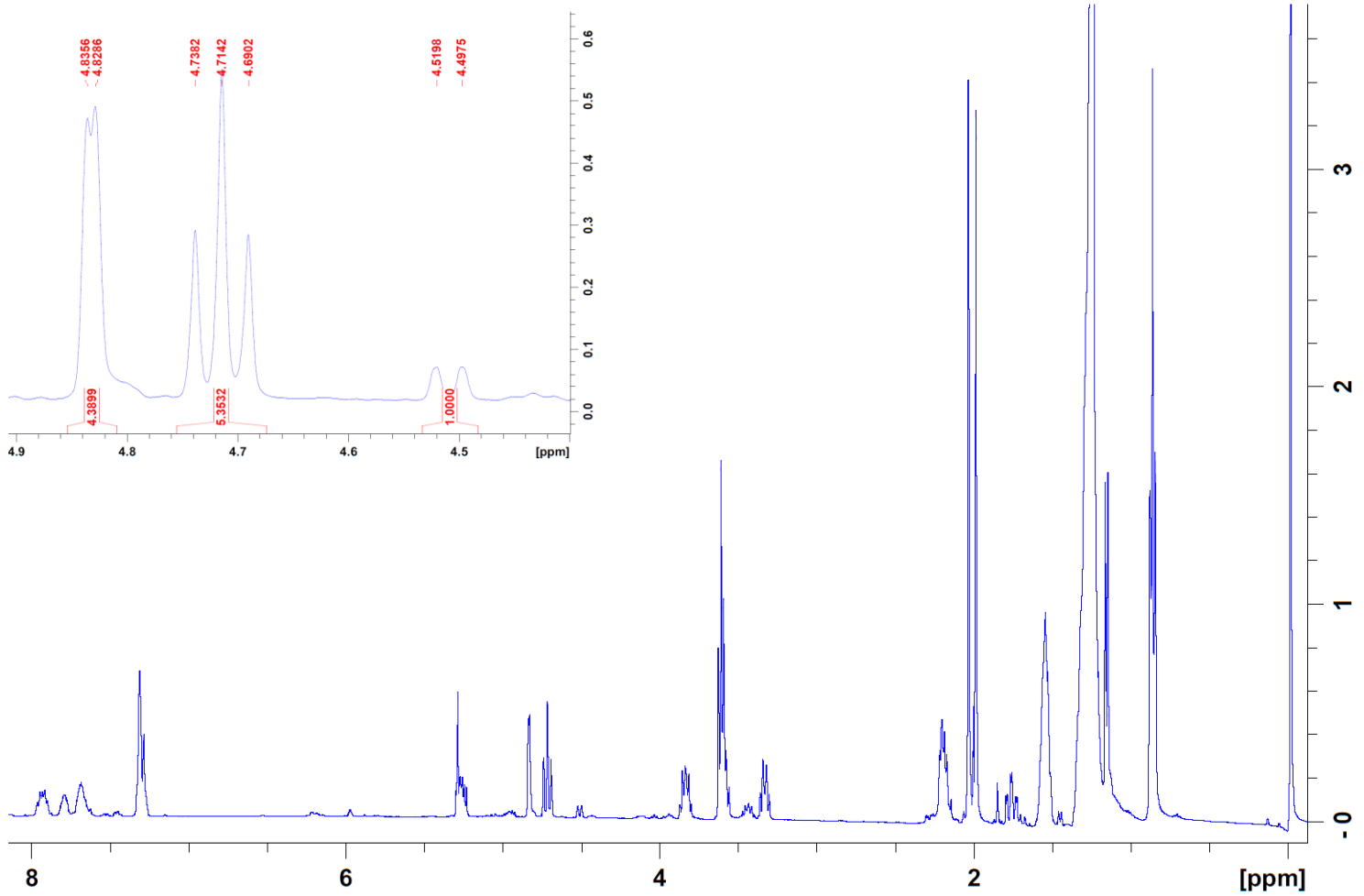
Annex 2. B - NMR from reactional system a, in CH_3CN



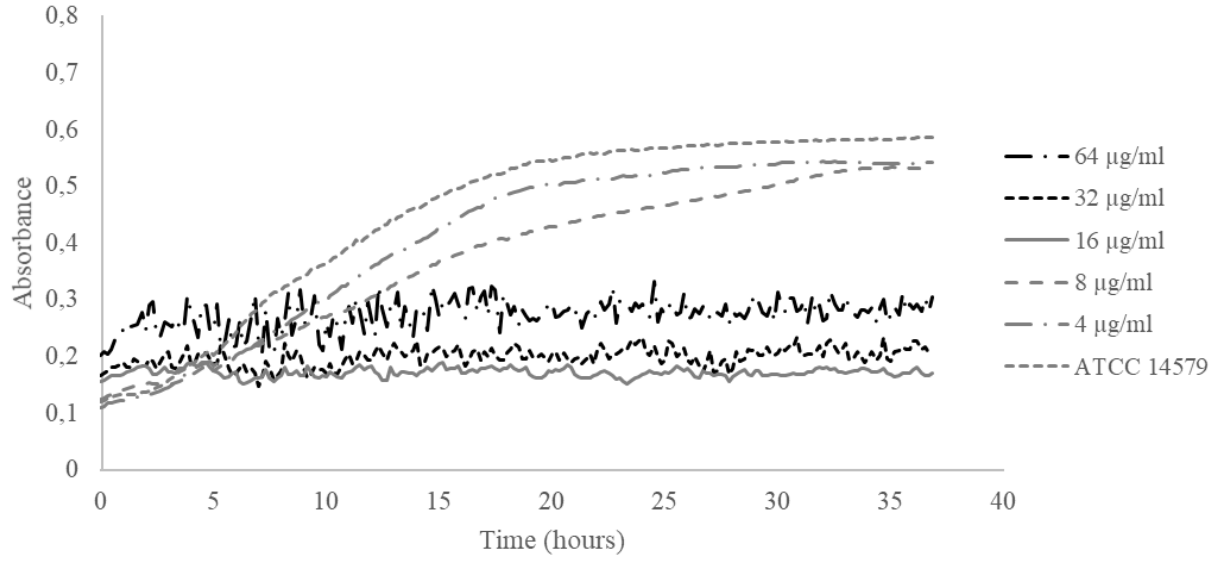
Annex 2. C - NMR from reactional system b, in CH_2Cl_2

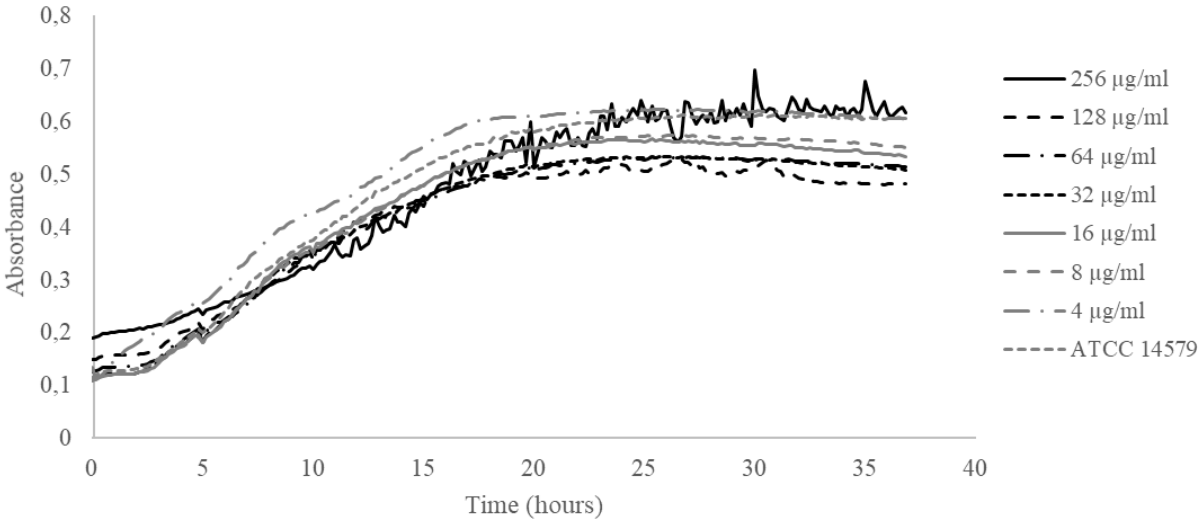


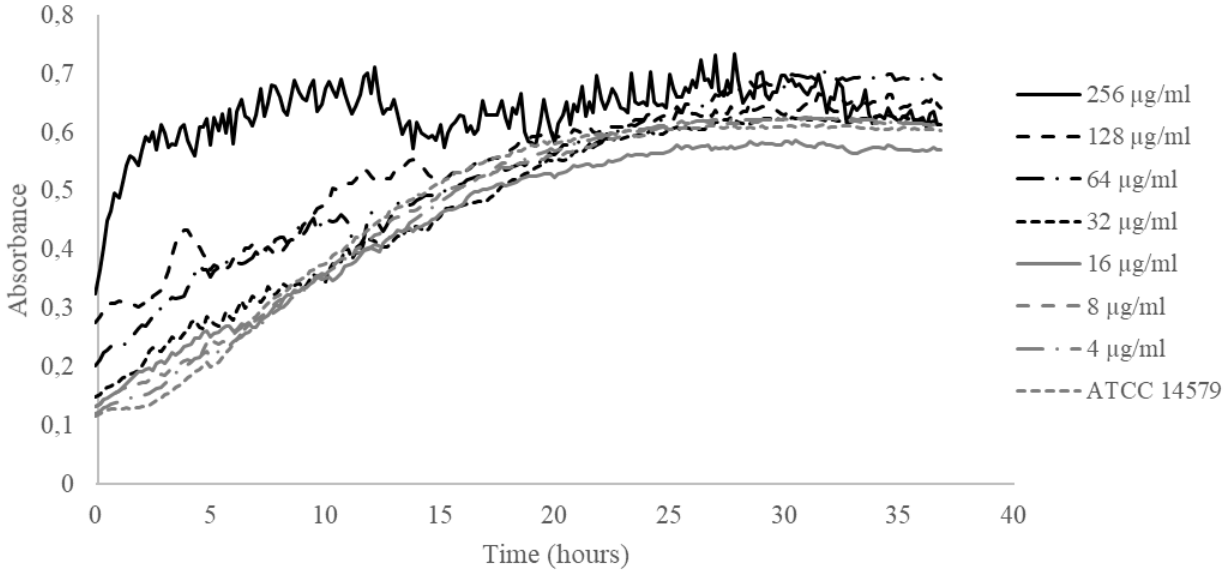
Annex 2. D - NMR from reactional system c, in CH_3CN



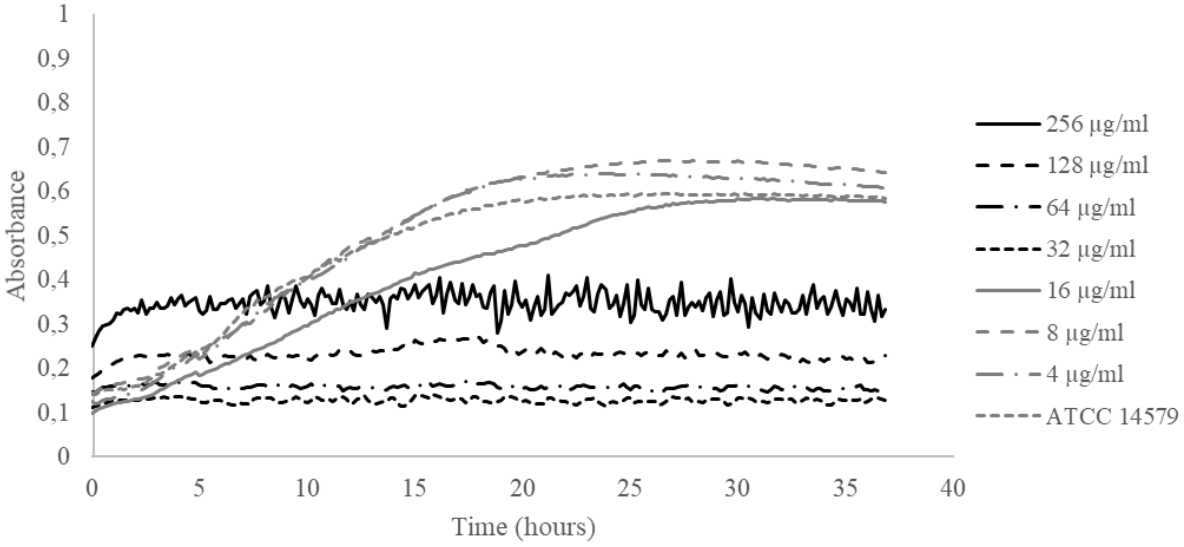
Annex 3. Bacterial growth curves for *B. cereus* ATCC 14579 in the presence of compounds **1**, **4** and **8**, respectively

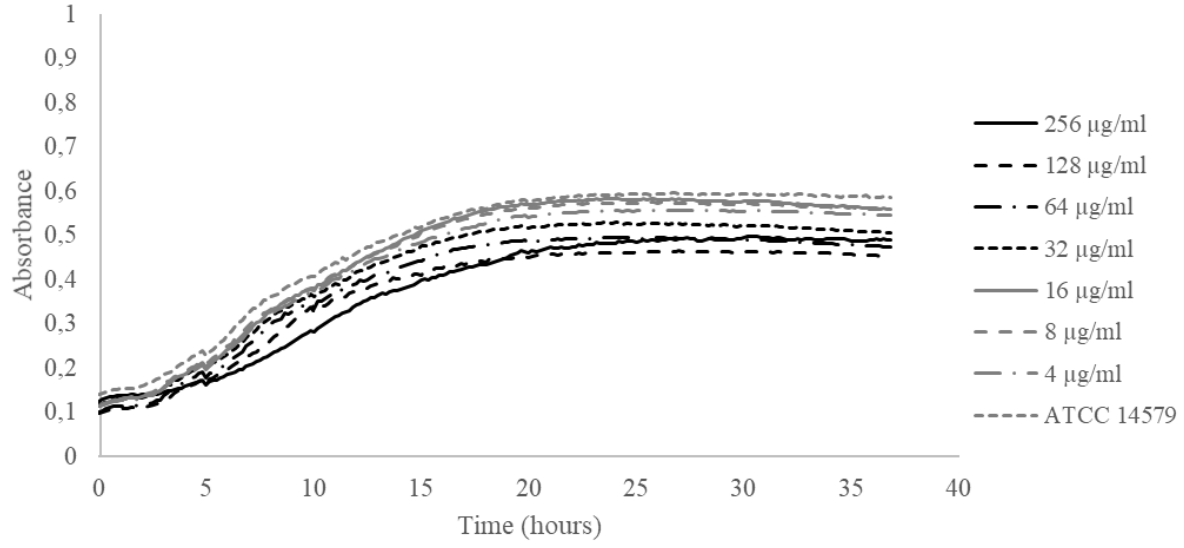


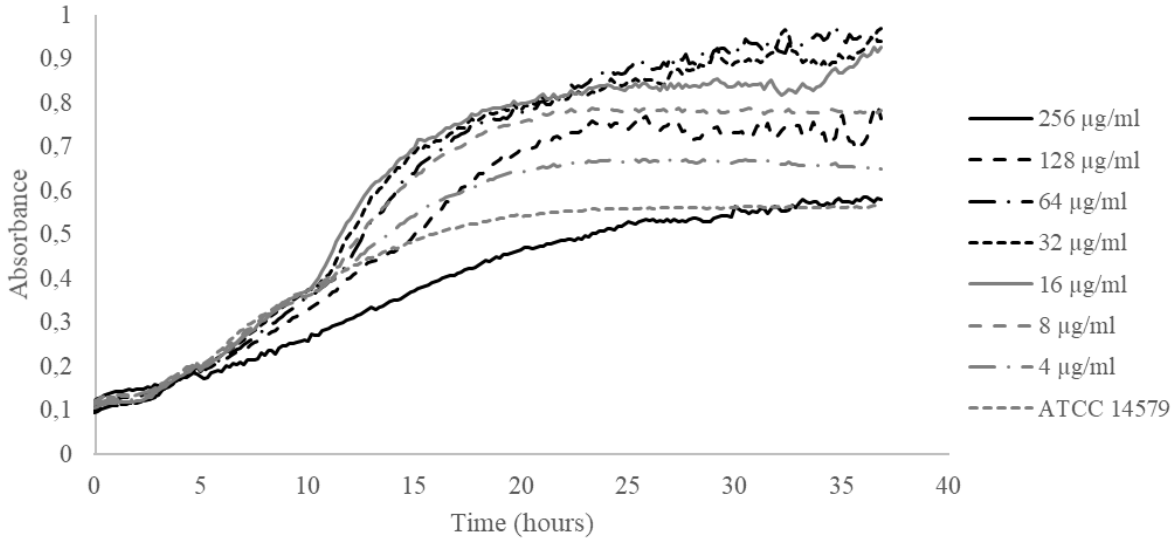



0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
Absorbance
0
5
10
15
20
25
30
35
40
Time (hours)
256 µg/ml
128 µg/ml
64 µg/ml
32 µg/ml
16 µg/ml
8 µg/ml
4 µg/ml
ATCC 14579

Annex 4. Bacterial growth curves for *B. cereus* ATCC 14579 in the presence of compounds **11**, **13** and **14**, respectively





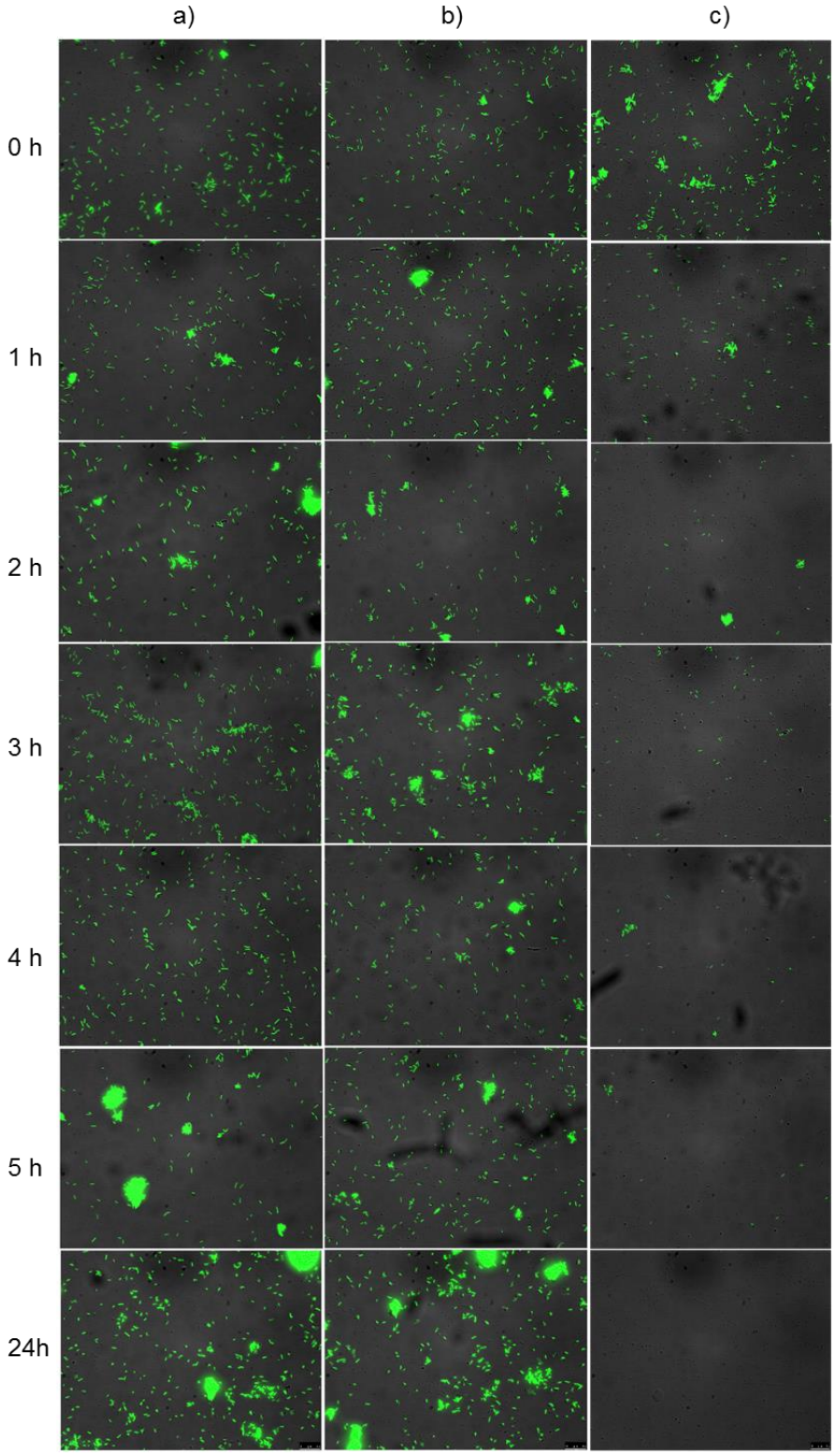

Absorbance
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
0
5
10
15
20
25
30
35
40
Time (hours)
256 µg/ml
128 µg/ml
64 µg/ml
32 µg/ml
16 µg/ml
8 µg/ml
4 µg/ml
ATCC 14579

Annex 5. Images obtained from different temporal points, in 3 experimental conditions:

a) Bacterial culture

b) Bacterial culture with DMSO 6%

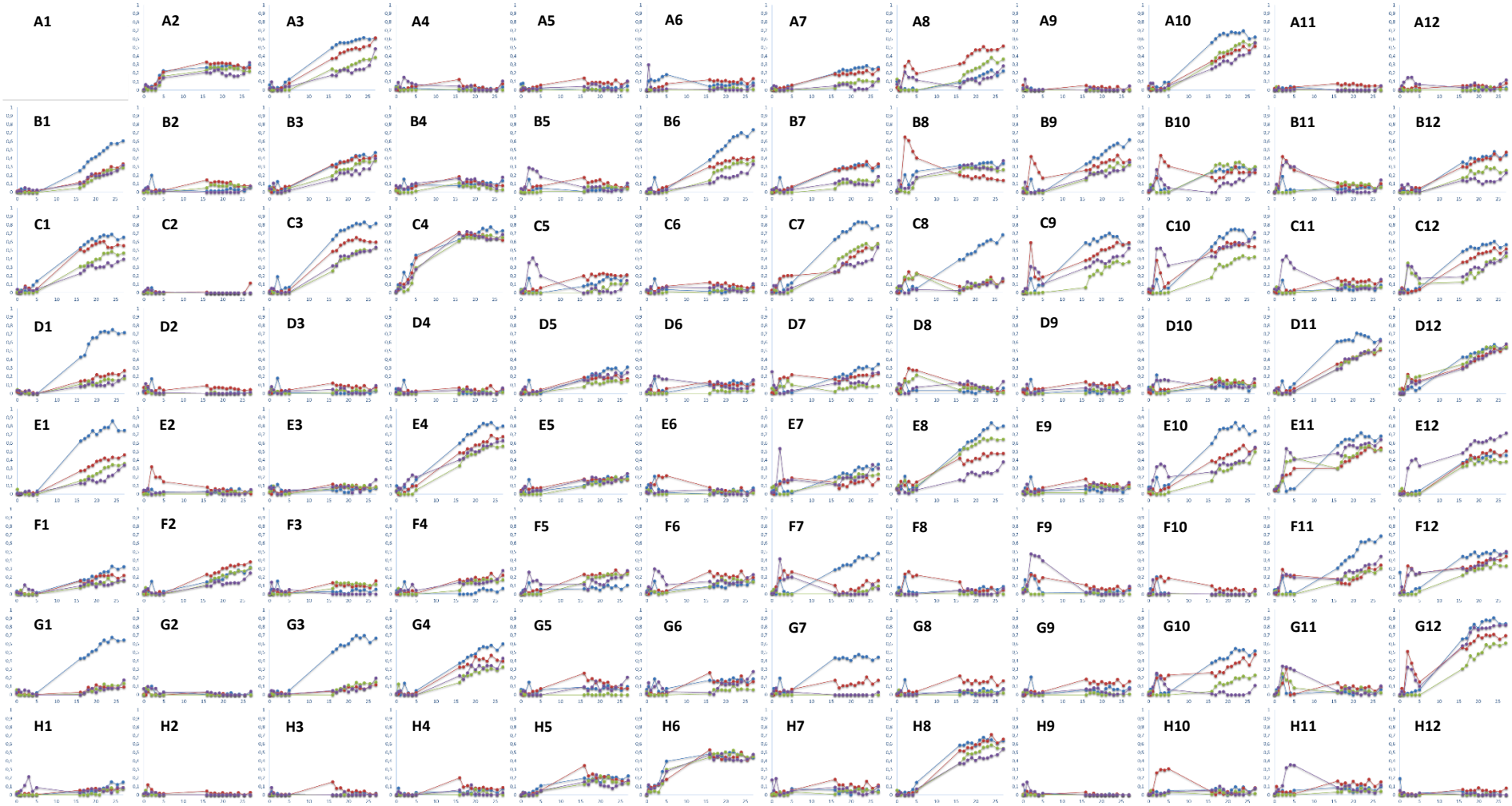
c) Bacterial culture with compound **1** [256 µg/mL]



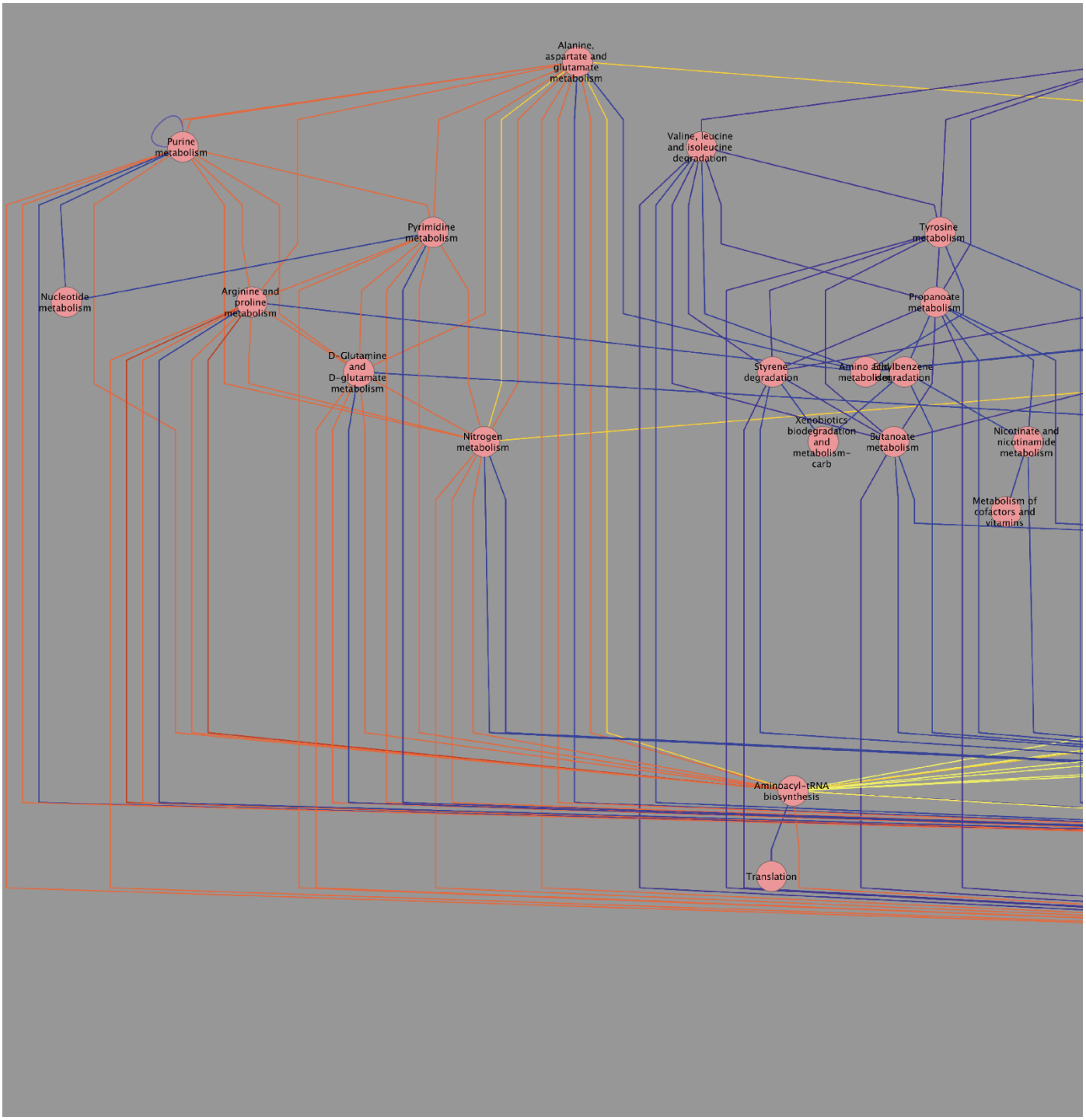
Annex 6. Carbon sources of PM1 plate used for phenotypic assay

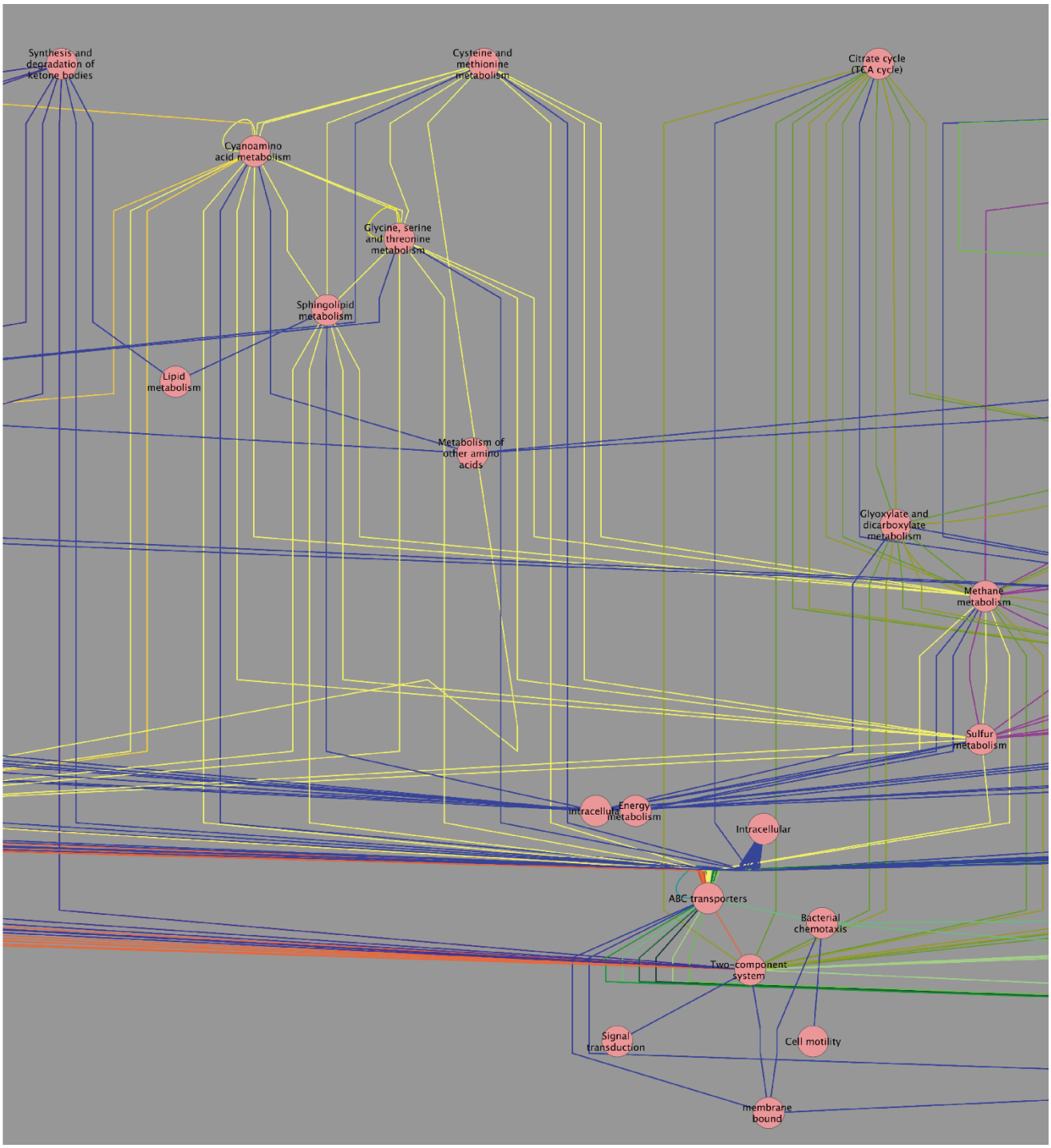
A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D-Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Glucuronic Acid	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α-D-Glucose	C10 Maltose	C11 D-Melibiose	C12 Thymidine
D-1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid	D8 α-Methyl-D-Galactoside	D9 α-D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 m-Tartaric Acid	E3 D-Glucose-1-Phosphate	E4 D-Fructose-6-Phosphate	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 β-Methyl-D-Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 m-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L-Glutamic Acid	G2 Tricarballylic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid	G8 N-Acetyl-β-D-Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 p-Hydroxy Phenyl Acetic Acid	H3 m-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose	H6 L-Lyxose	H7 Glucuronamide	H8 Pyruvic Acid	H9 L-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

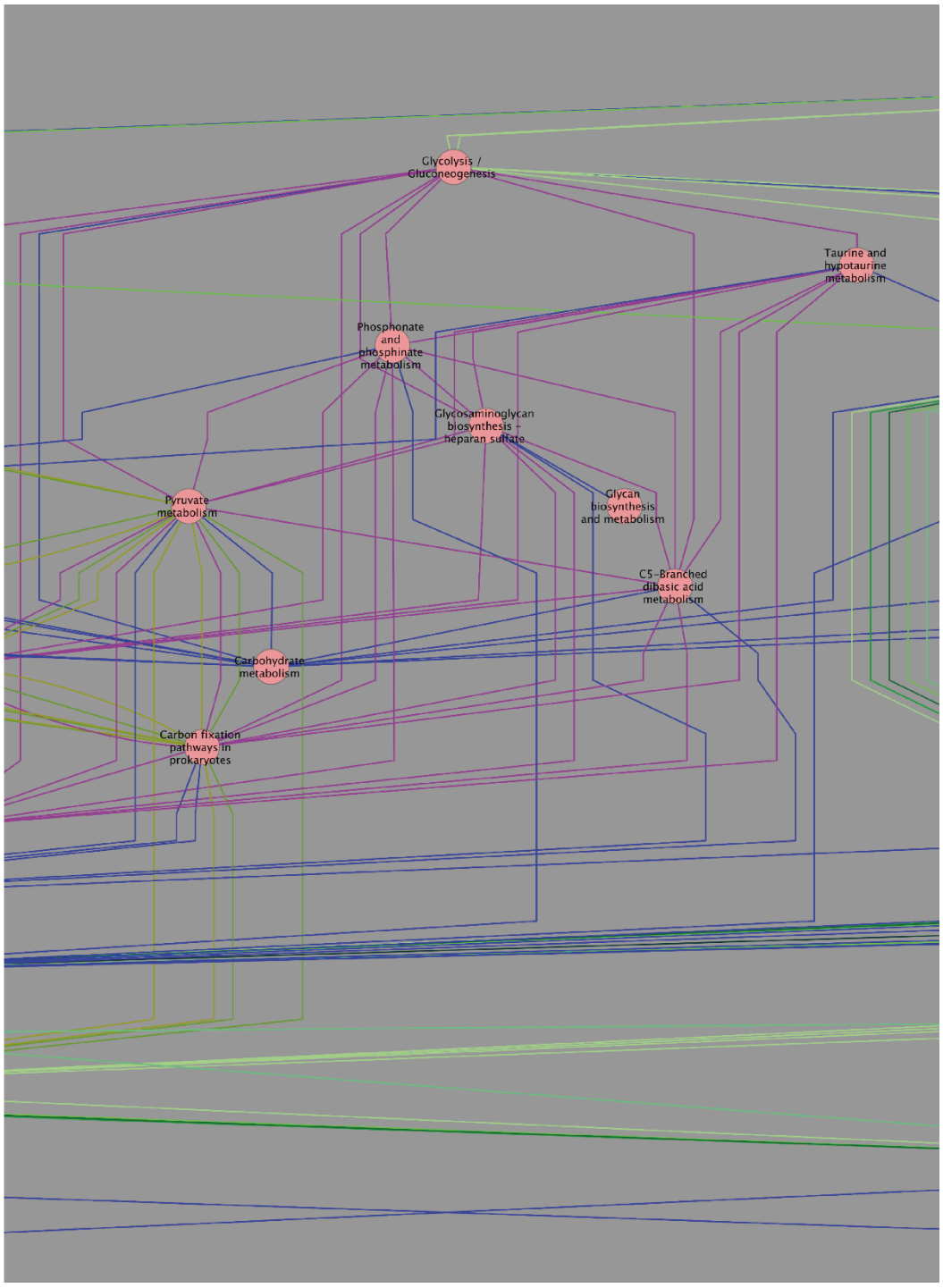
Annex 7. Impact of compound **1** in the metabolism of *B. cereus* ATCC 14579 with diverse single carbon sources

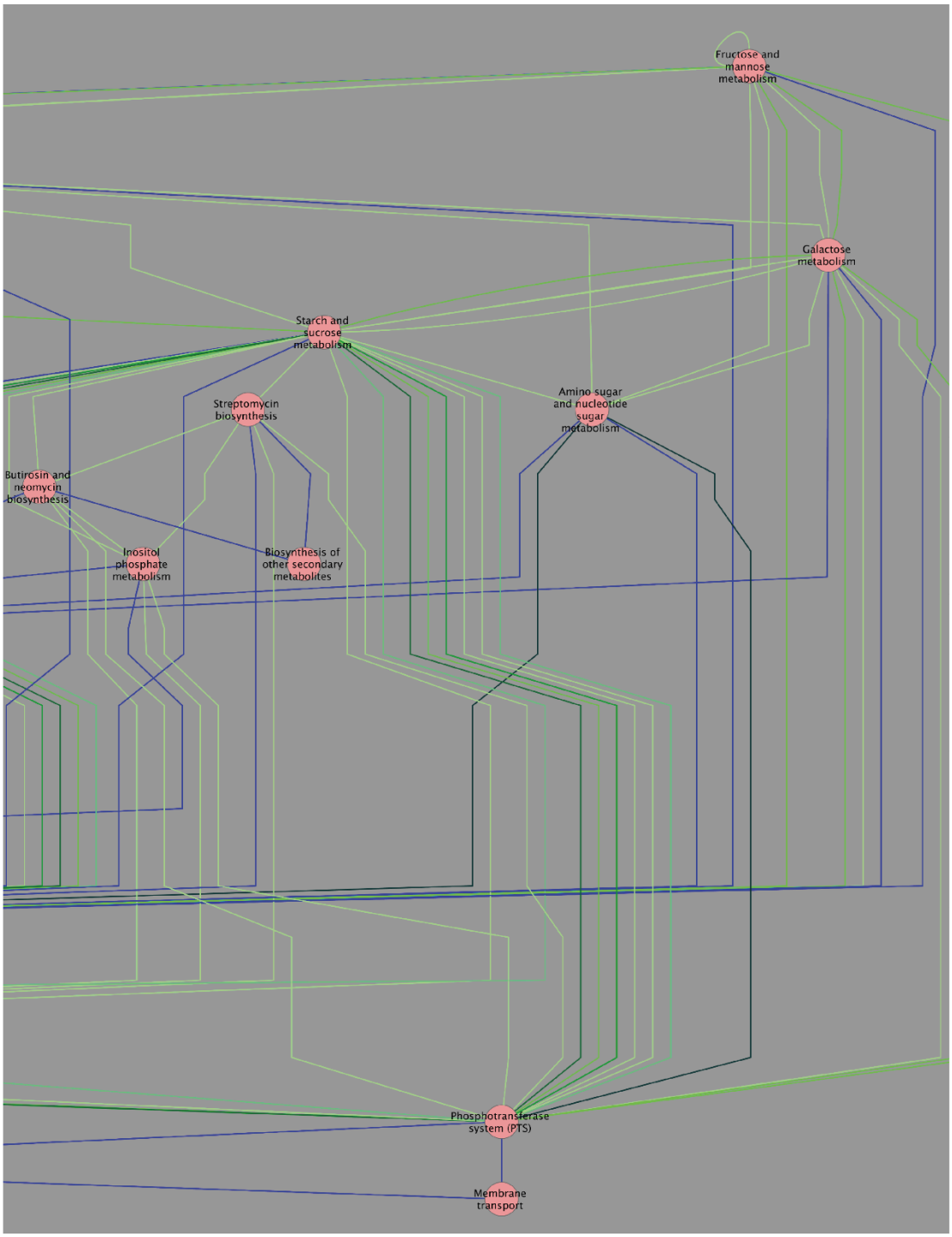


Annex 8. Complete metabolic reconstruction map

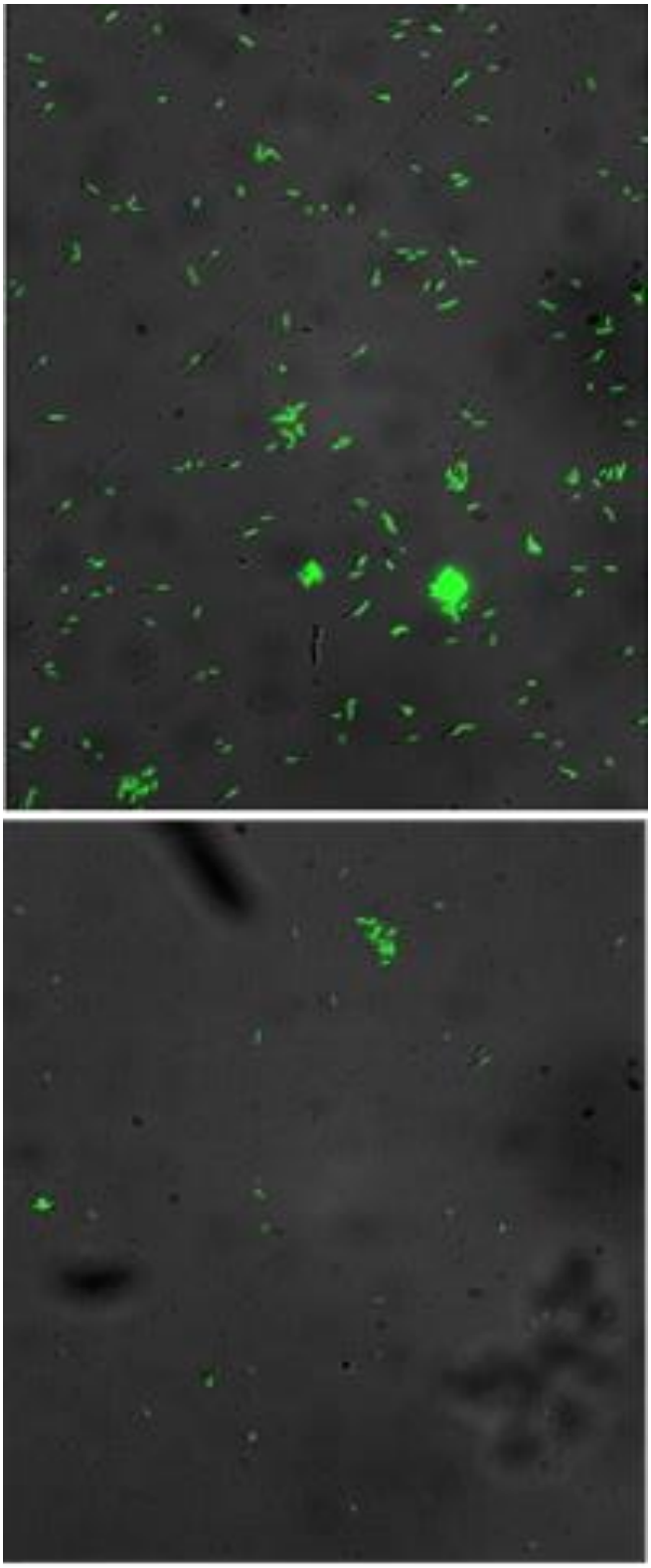



Synthesis and degradation of ketone bodies
Cysteine and methionine metabolism
Citrate cycle (TCA cycle)
Cyanoamino acid metabolism
Glycine, serine and threonine metabolism
Sphingolipid metabolism
Lipid metabolism
Metabolism of other amino acids
Glyoxylate and dicarboxylate metabolism
Methane metabolism
Sulfur metabolism
Intracellula
Energy metabolism
Intracellular
ABC transporters
Bacterial chemotaxis
Two-component system
Signal transduction
Cell motility
membrane bound

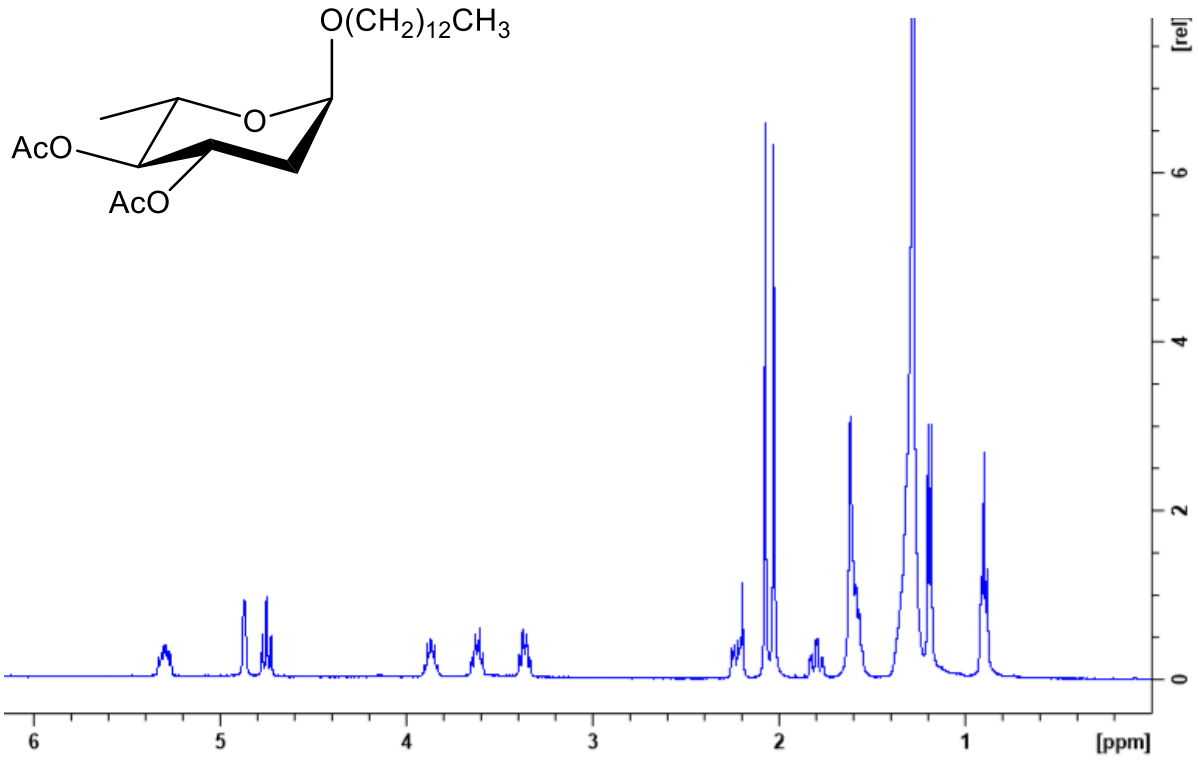

Glycolysis /
Gluconeogenesis
Taurine and
hypotaurine
metabolism
Phosphonate
and
phosphinate
metabolism
Glycosaminoglycan
biosynthesis -
heparan sulfate
Pyruvate
metabolism
Glycan
biosynthesis
and metabolism
C5-Branched
dibasic acid
metabolism
Carbohydrate
metabolism
Carbon fixation
pathways in
prokaryotes

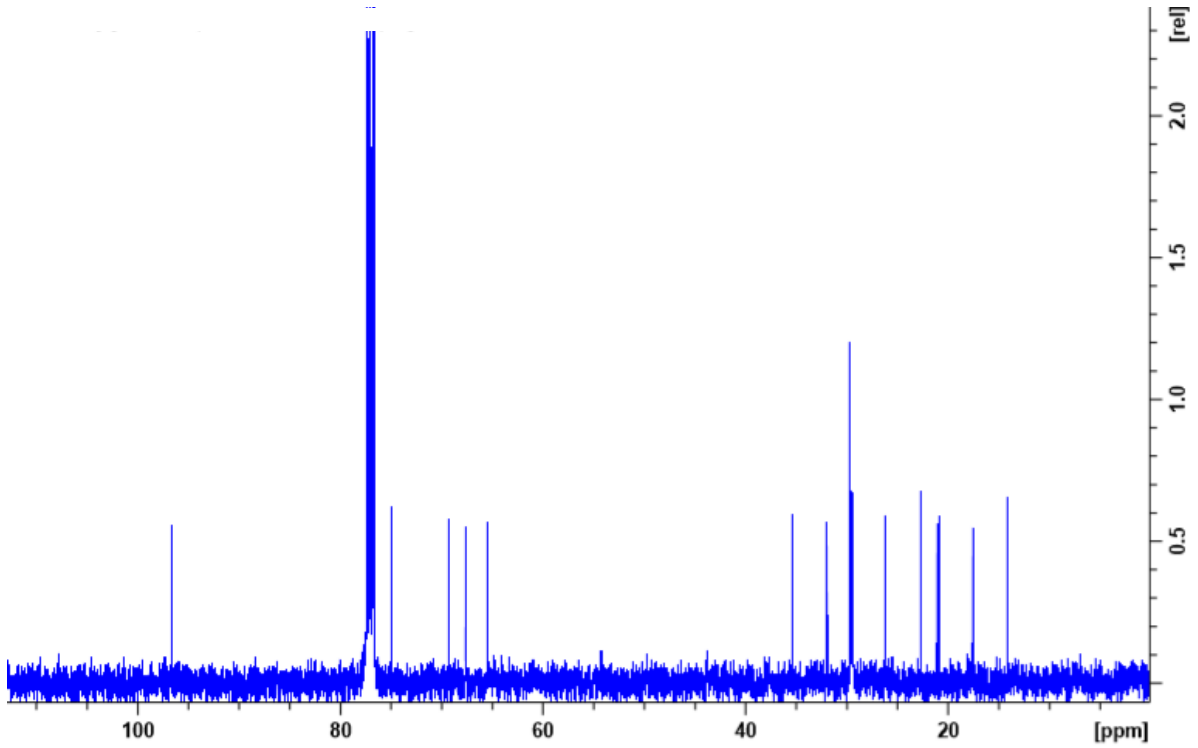

Fructose and mannose metabolism
Galactose metabolism
Starch and sucrose metabolism
Streptomycin biosynthesis
Amino sugar and nucleotide sugar metabolism
Butirosin and neomycin biosynthesis
Inositol phosphate metabolism
Biosynthesis of other secondary metabolites
Phosphotransferase system (PTS)
Membrane transport

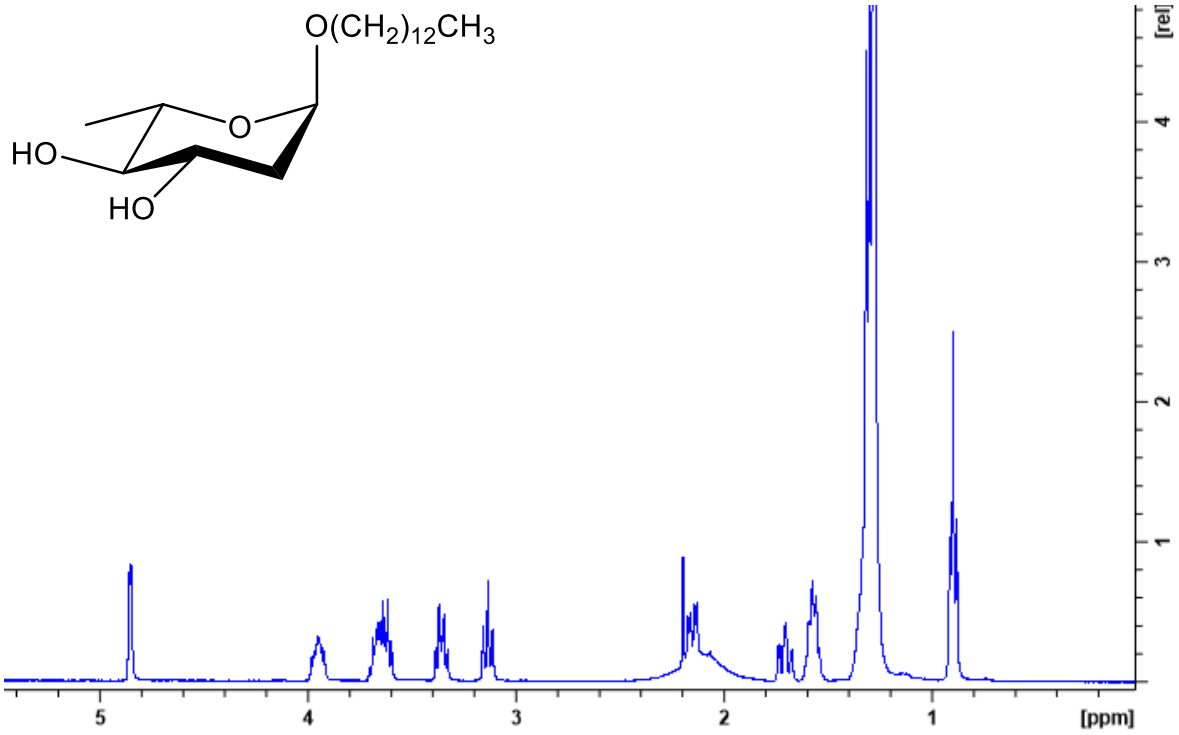
Annex 9. Comparison of sample microscopy images of GFP fluorescence in a control condition (top) and after 4 hours of exposure to compound 1 [256 µg/mL] (bottom)

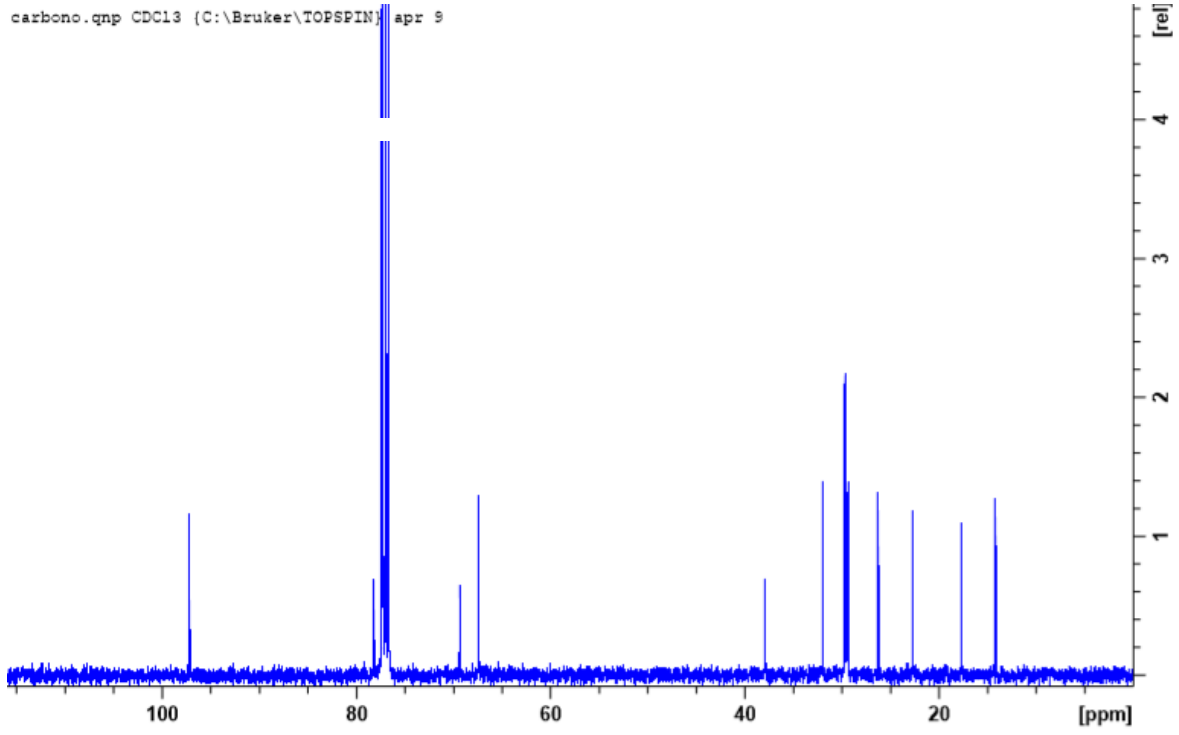


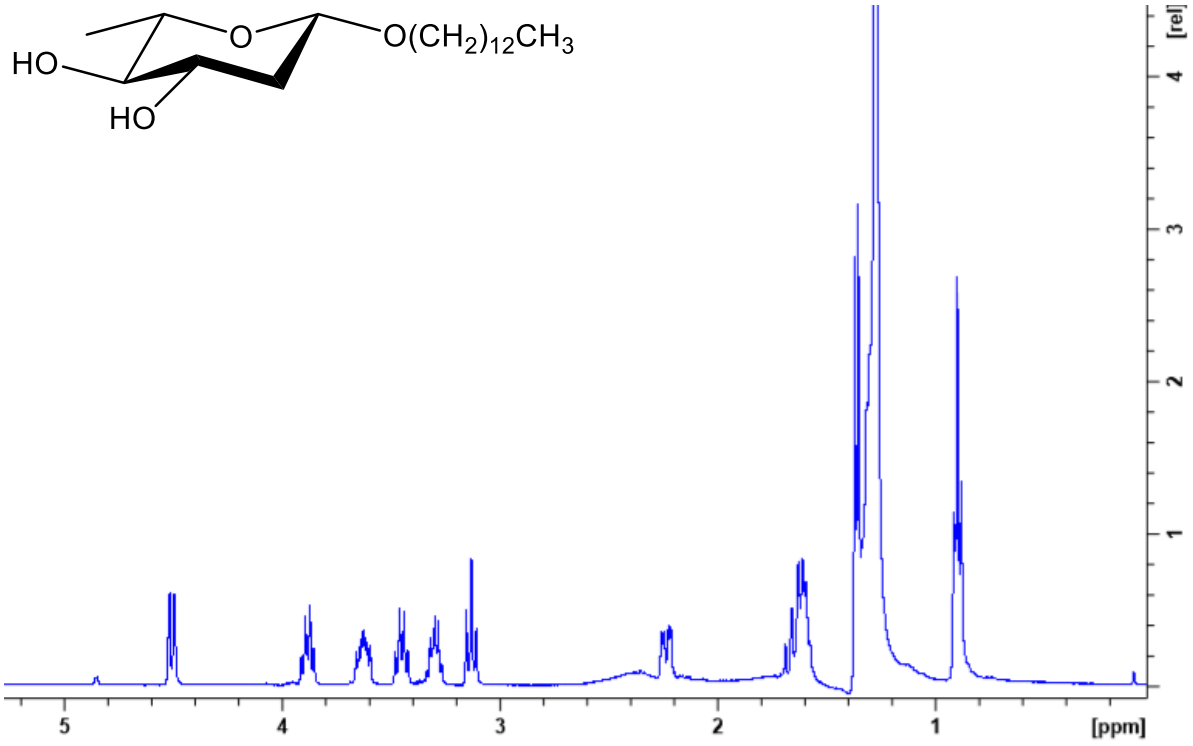
Annex 10. NMR data relative to the compounds sinthesized and isolated.

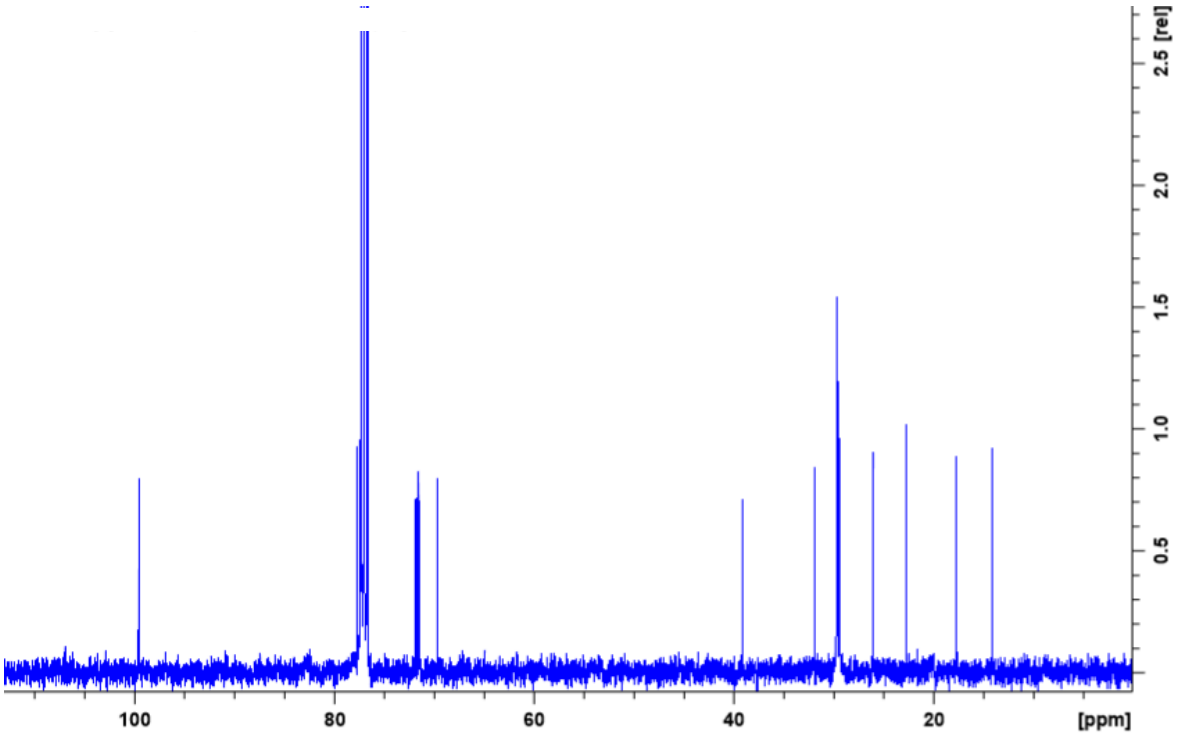


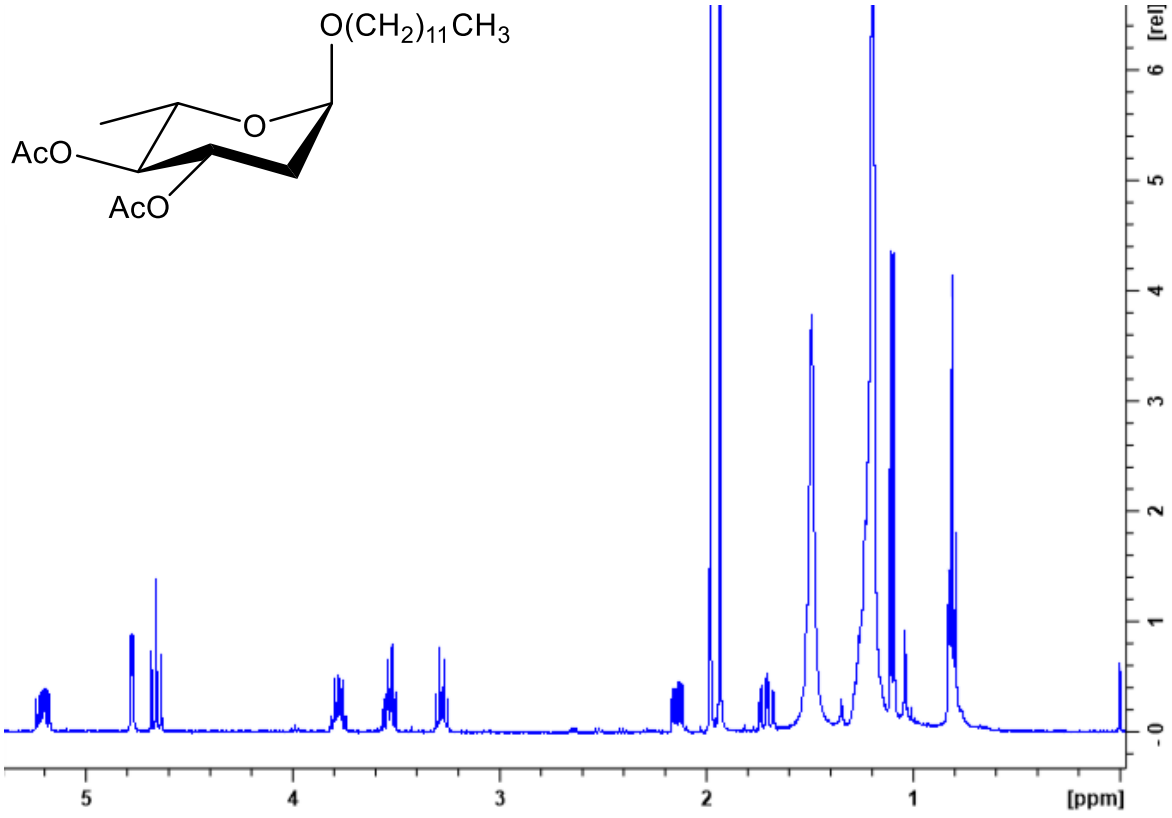


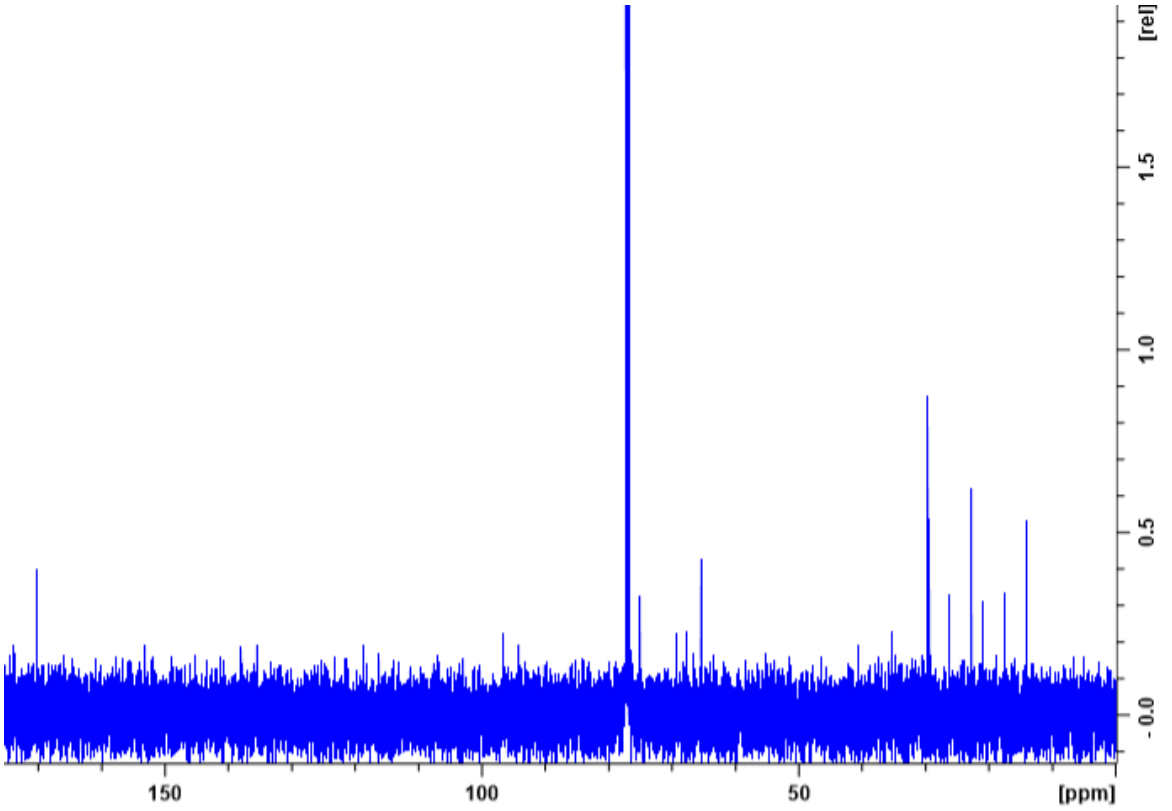

O(CH2)12CH3
O
HO
HO
[rel]
[ppm]

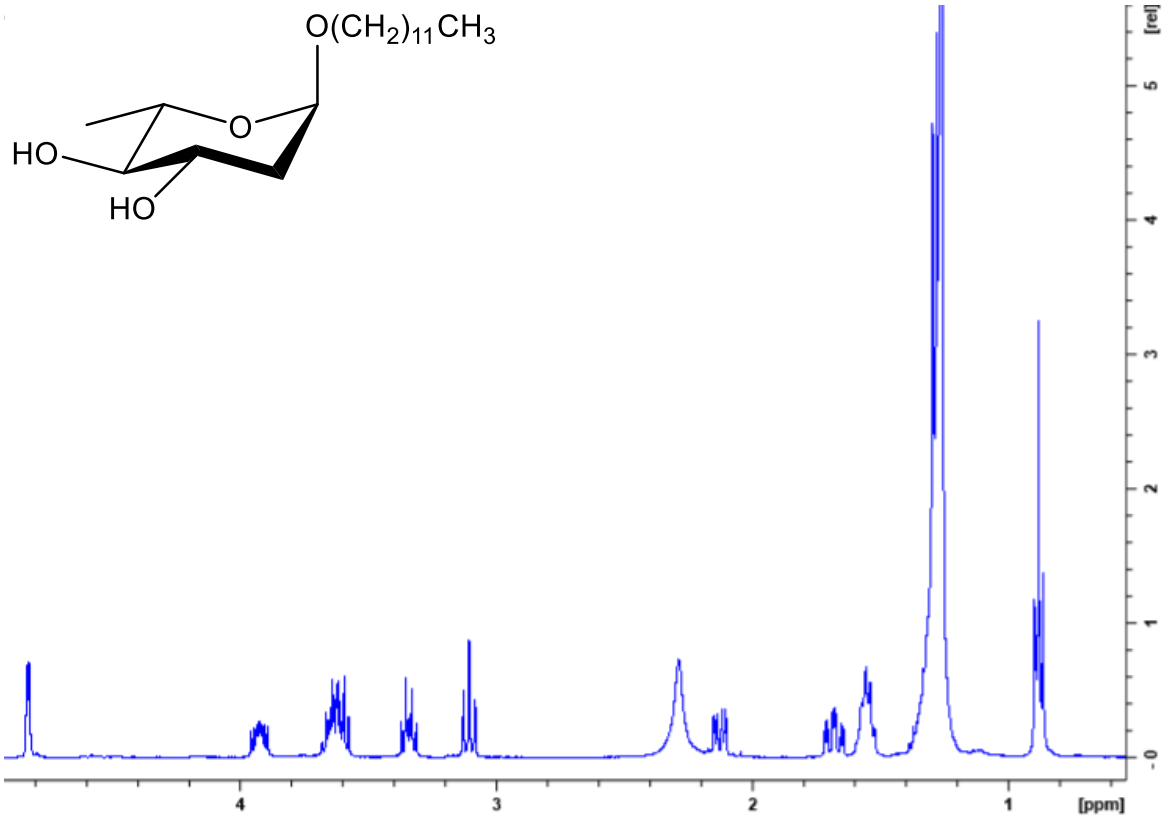

carbono.qnp CDCl3 {C:\Bruker\TOPSPIN} apr 9
[rel]
[ppm]

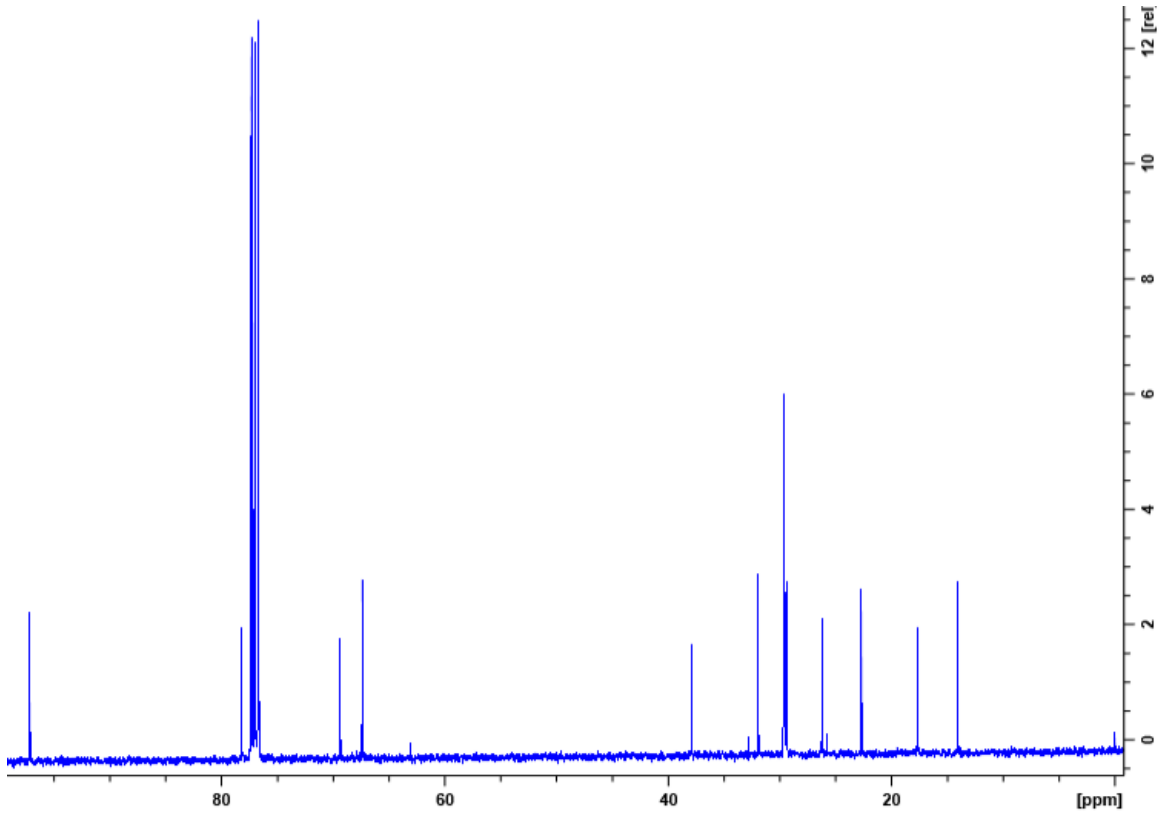

HO
HO
O
O(CH2)12CH3
5
4
3
2
1
[ppm]
1
2
3
4
[rel]

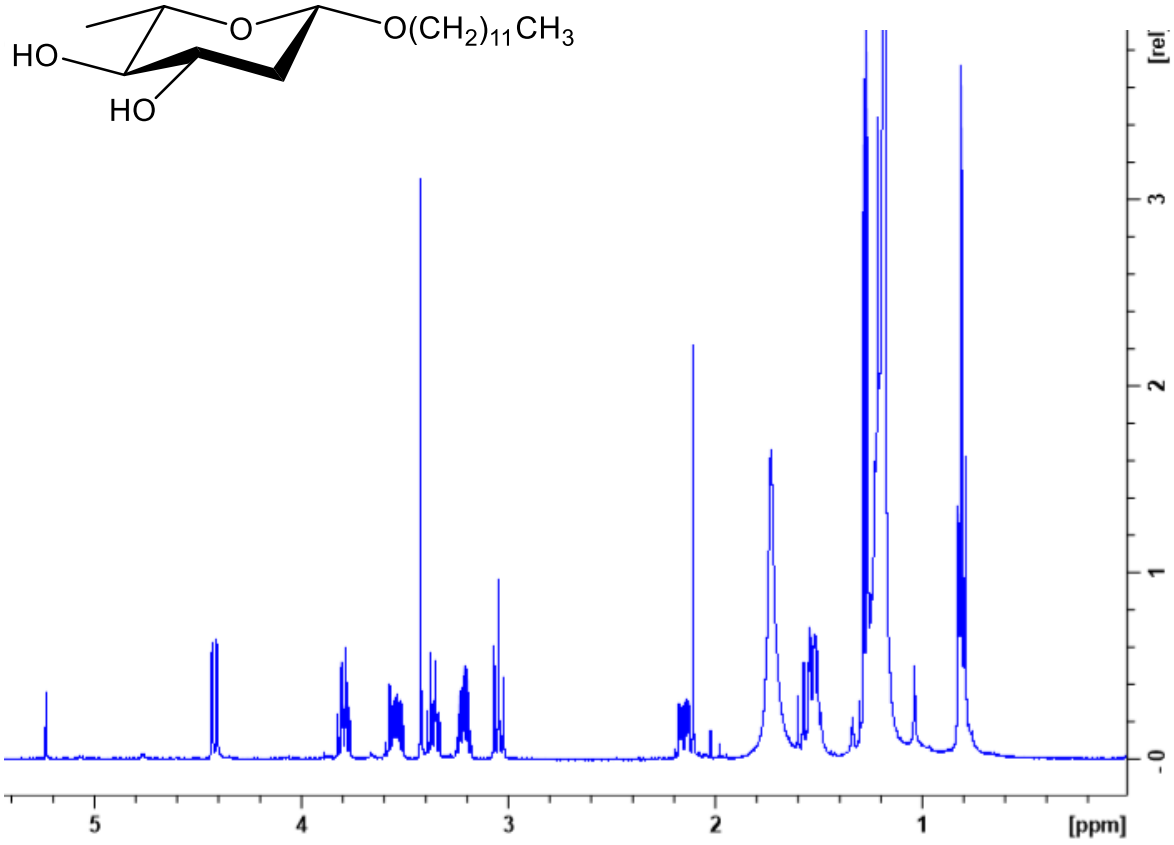

100
80
60
40
20
[ppm]
0.5
1.0
1.5
2.0
2.5 [rel]

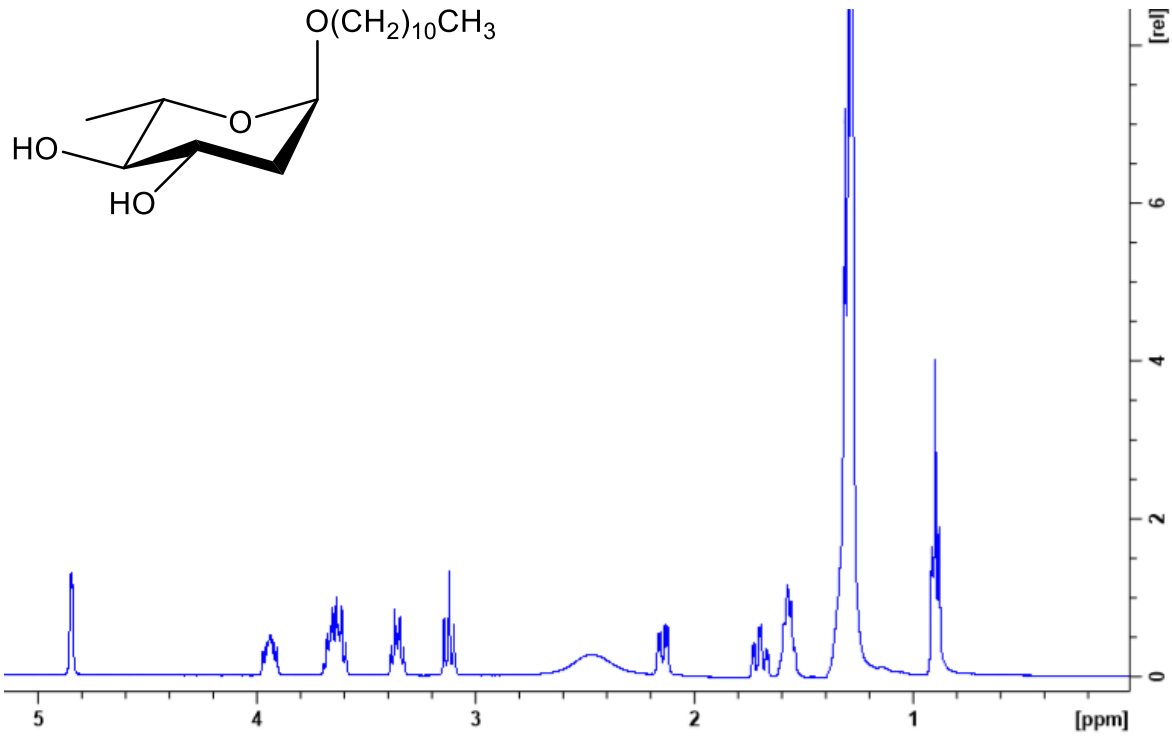

O(CH2)11CH3
O
AcO
AcO
[rel]
6
5
4
3
2
1
0
5
4
3
2
1
[ppm]

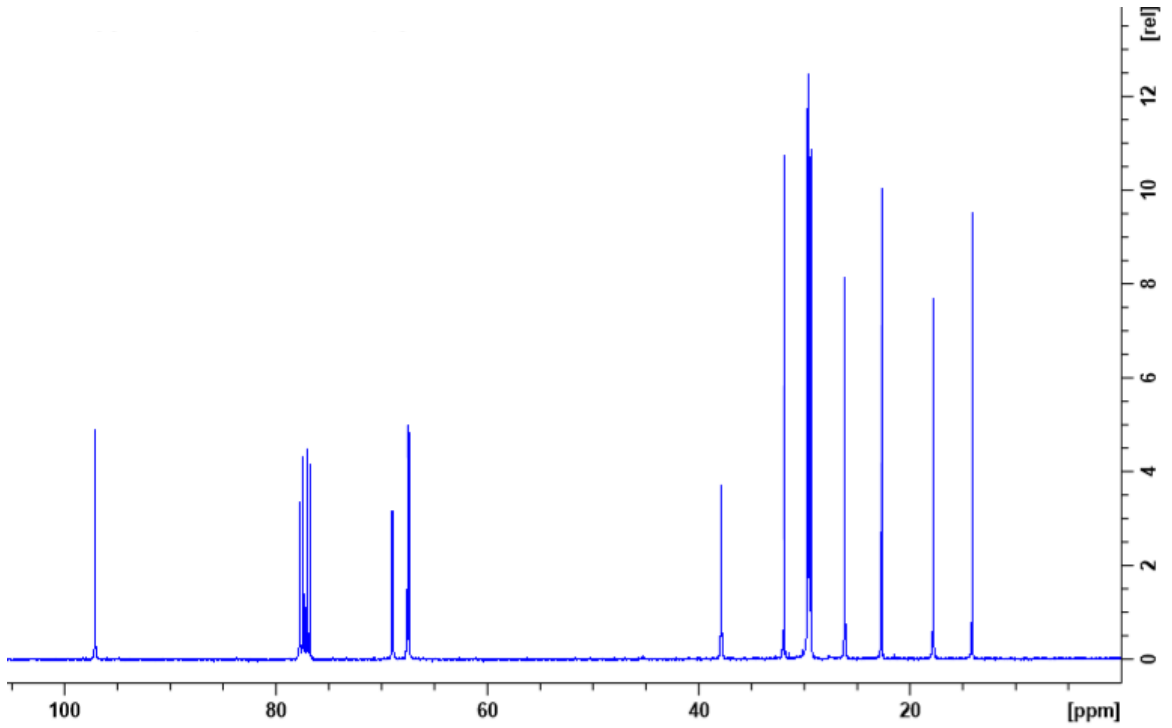

[rel]
1.5
1.0
0.5
0.0
150
100
50
[ppm]

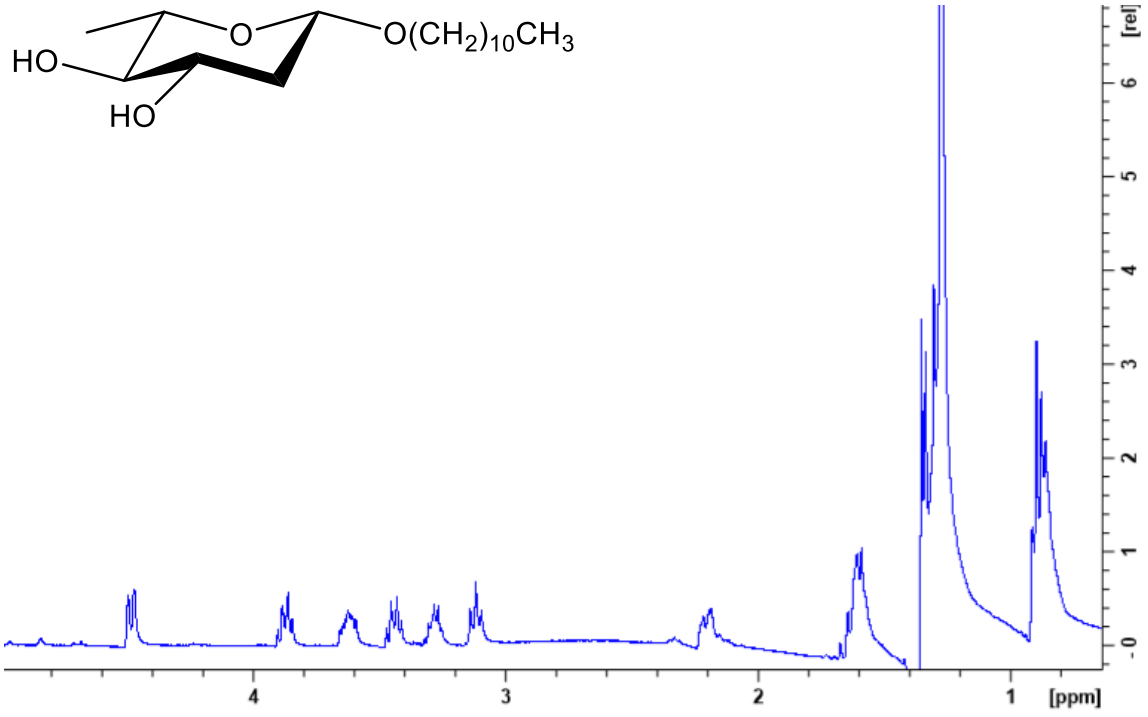

O(CH2)11CH3
O
HO
HO
[rel]
[ppm]

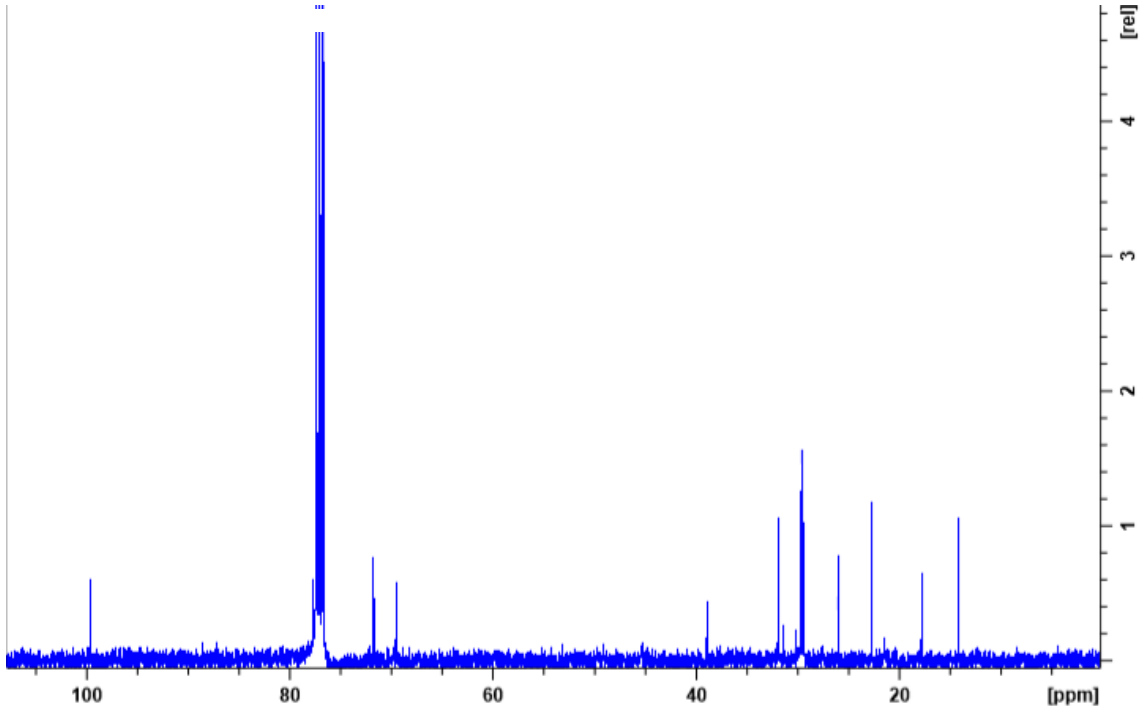

[rel]
[ppm]

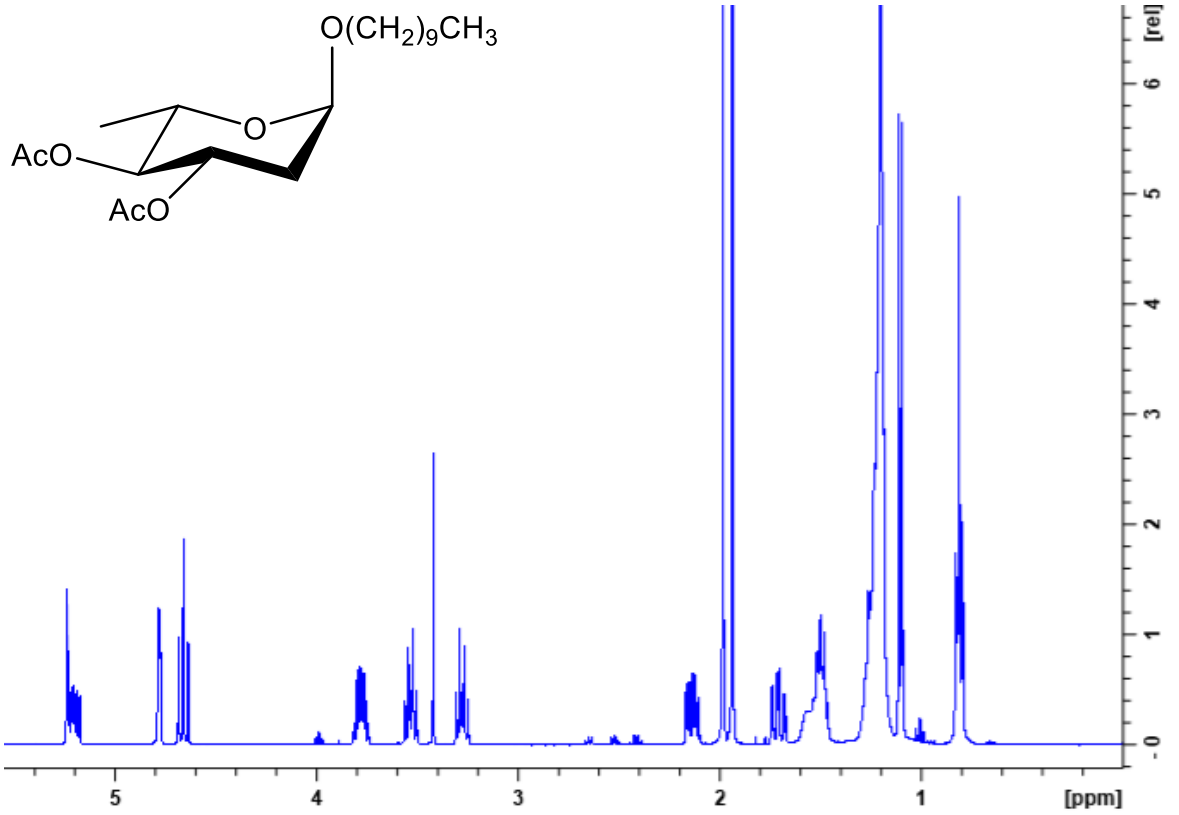

HO
O
O(CH2)11CH3
HO
[rel]
3
2
1
0
5
4
3
2
1
[ppm]

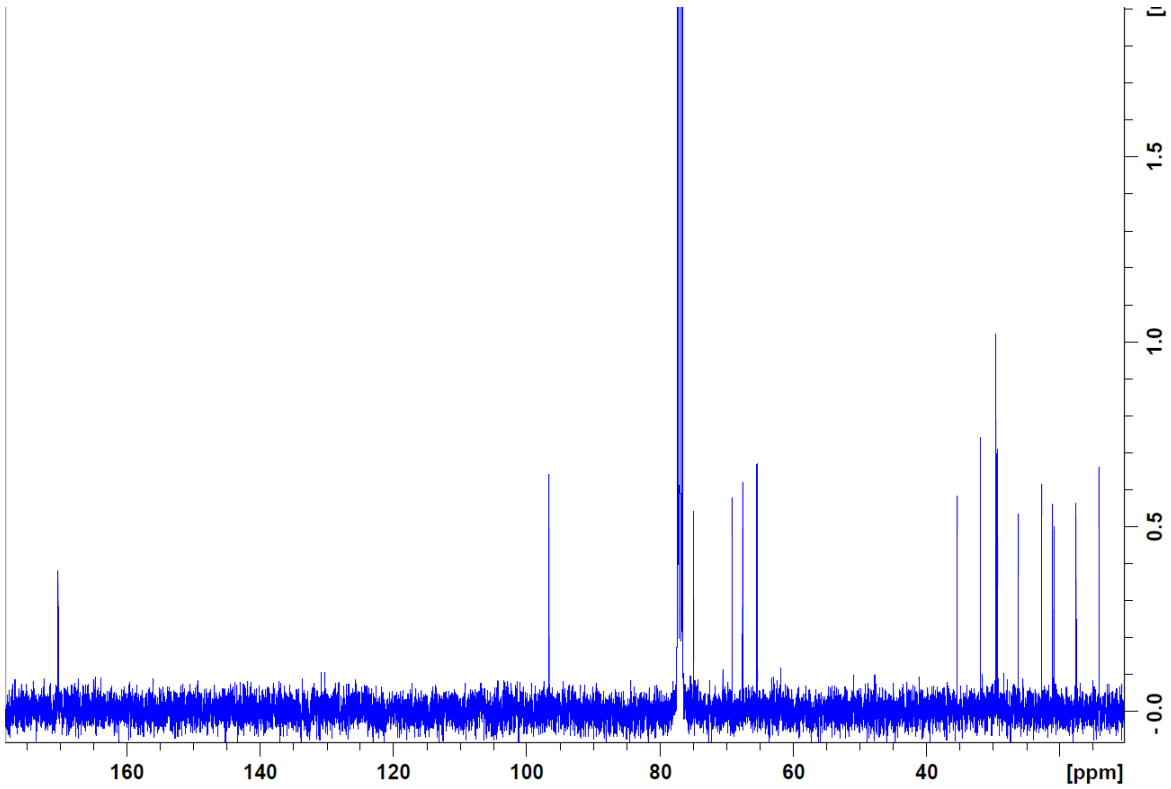

O(CH2)10CH3
O
HO
HO
[rel]
0
2
4
6
5
4
3
2
1
[ppm]

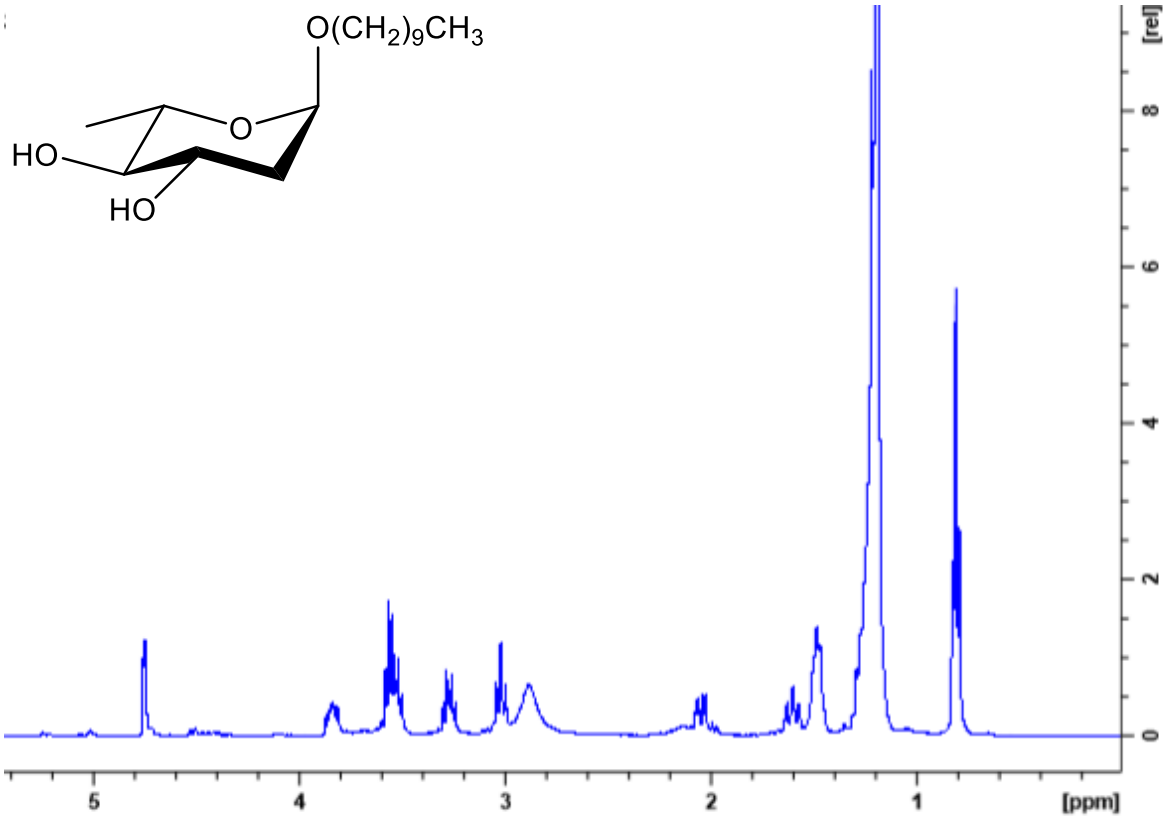

[rel]
0
2
4
6
8
10
12
100
80
60
40
20
[ppm]

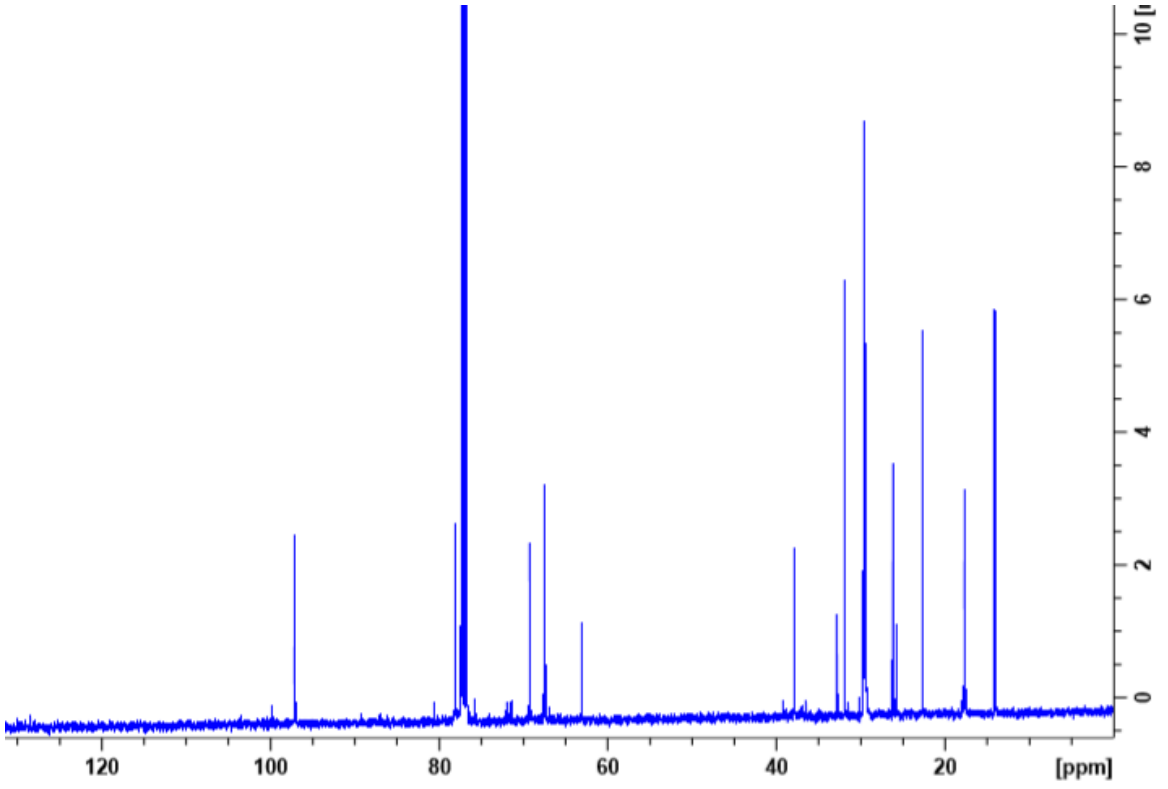

HO
O
O(CH2)10CH3
HO
[rel]
[ppm]

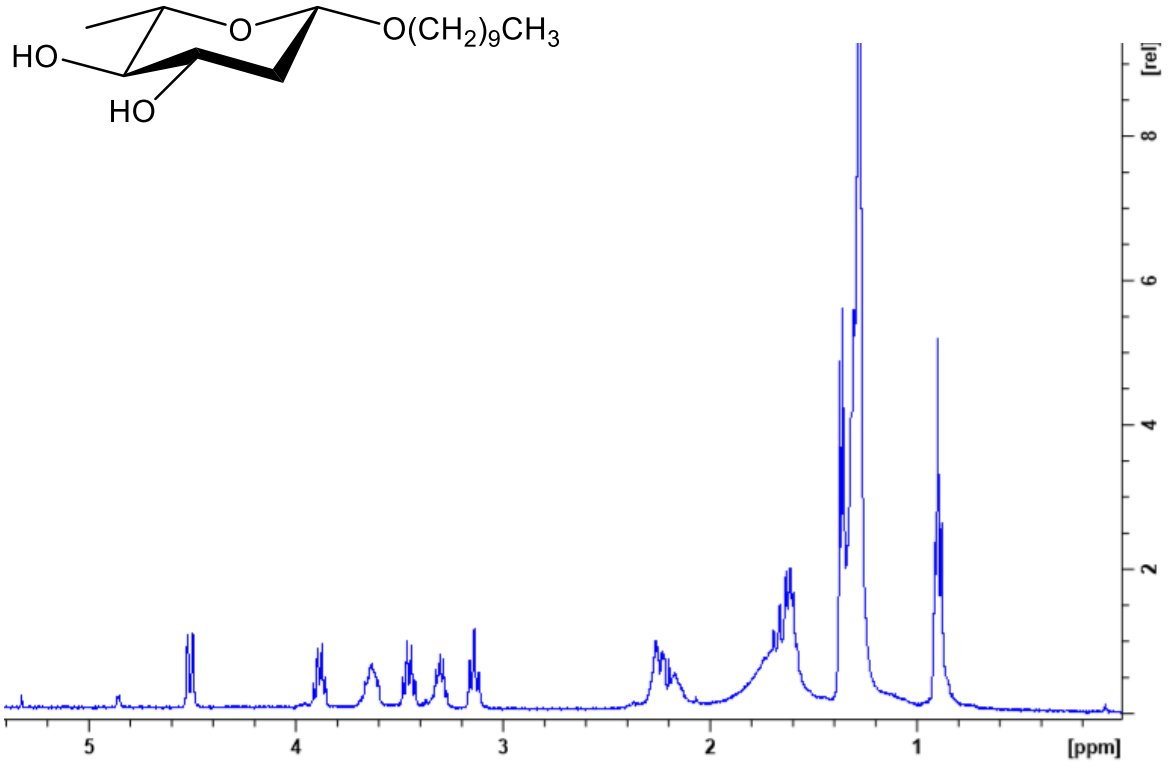

[rel]
[ppm]

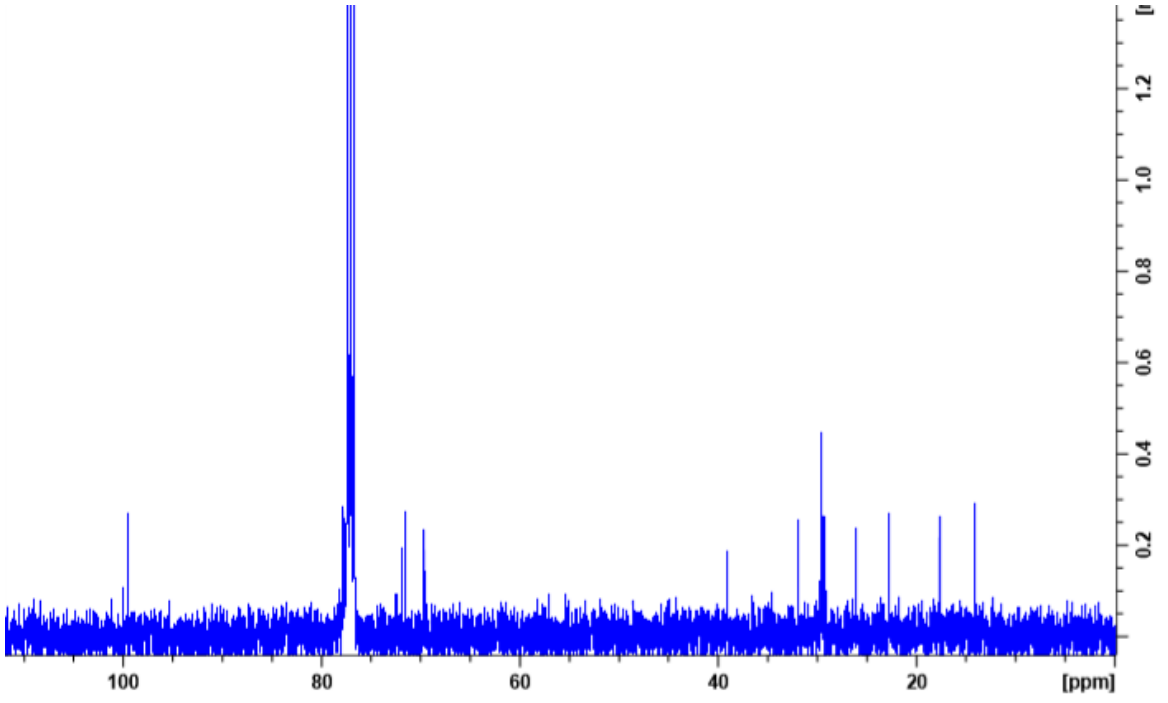

O(CH2)9CH3
O
AcO
AcO
[rel]
6
5
4
3
2
1
0
5
4
3
2
1
[ppm]

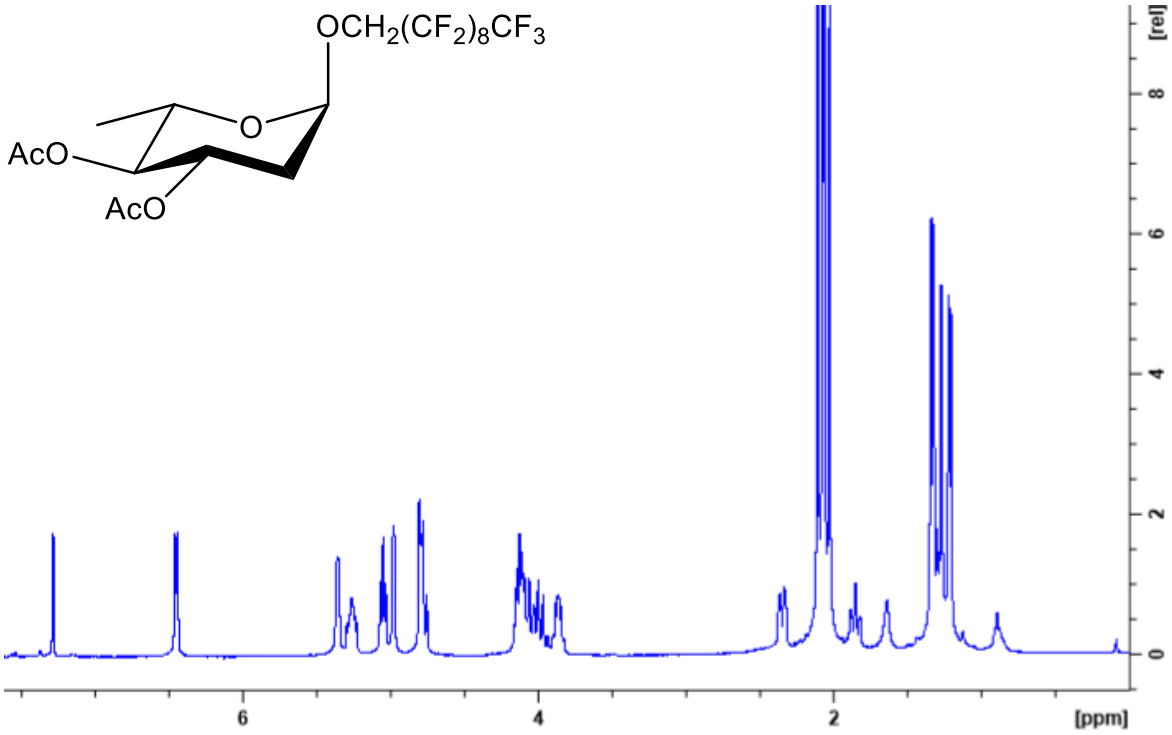

1.5
1.0
0.5
0.0
160
140
120
100
80
60
40
[ppm]

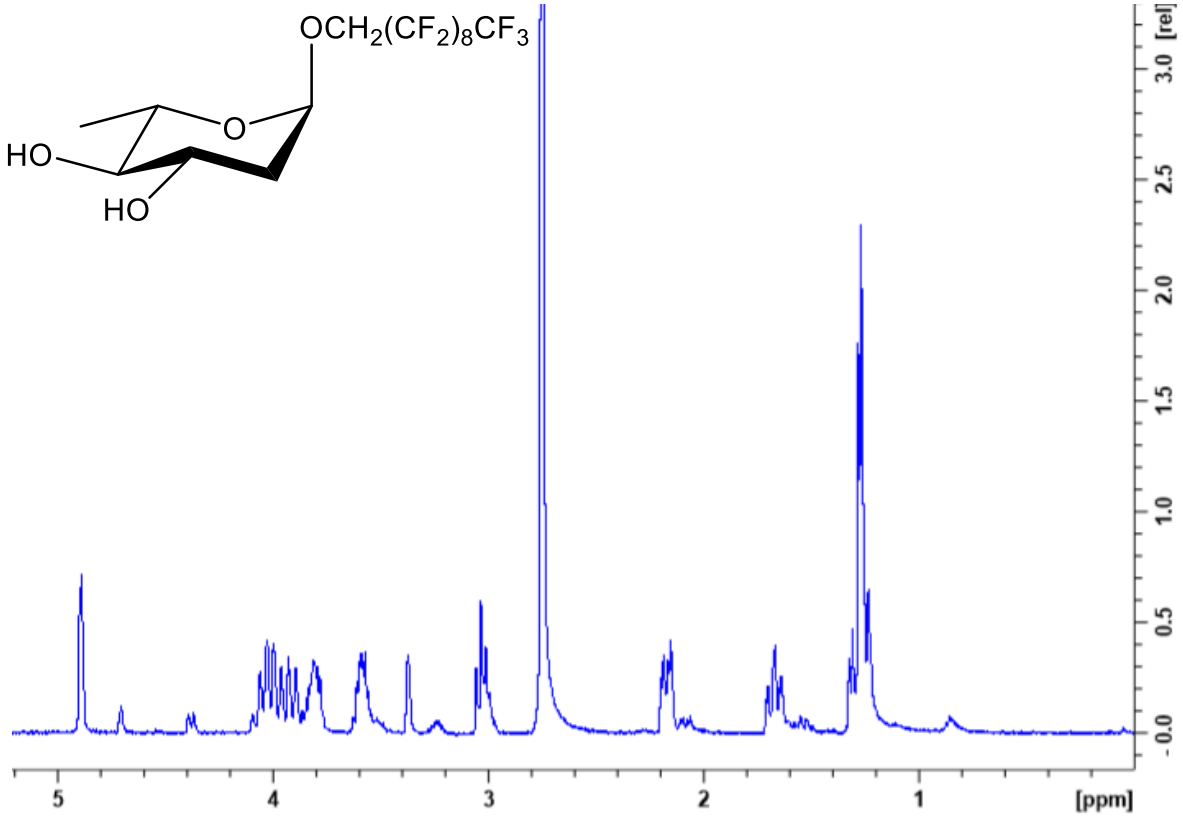

O(CH2)9CH3
O
HO
HO
[rel]
0
2
4
6
8
5
4
3
2
1
[ppm]

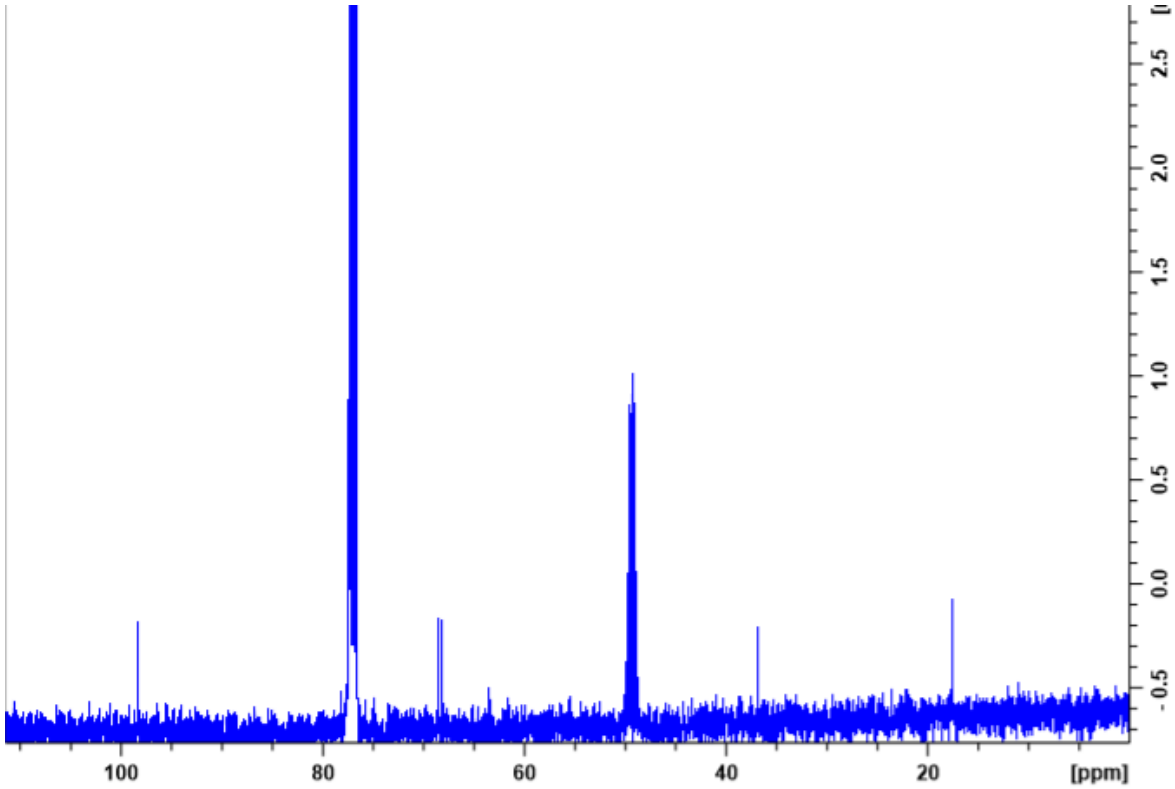

0
2
4
6
8
120
100
80
60
40
20
[ppm]

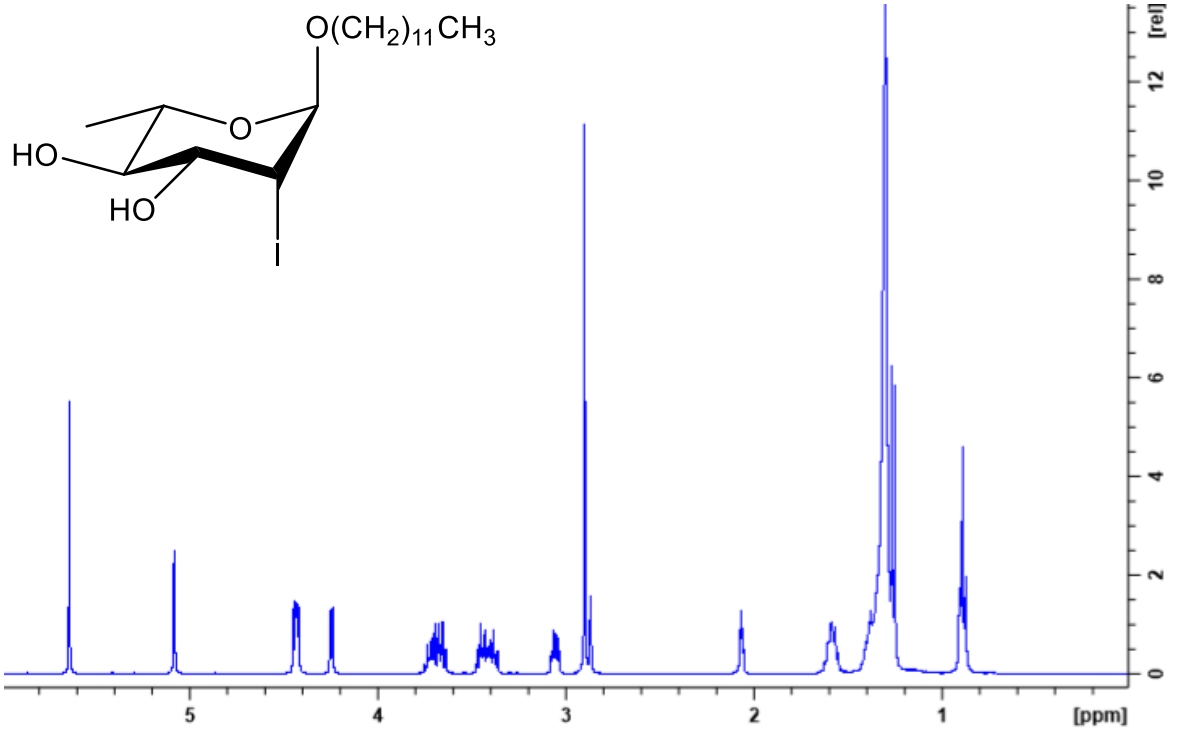

HO
O
O(CH2)9CH3
HO
[rel]
8
6
4
2
5
4
3
2
1
[ppm]

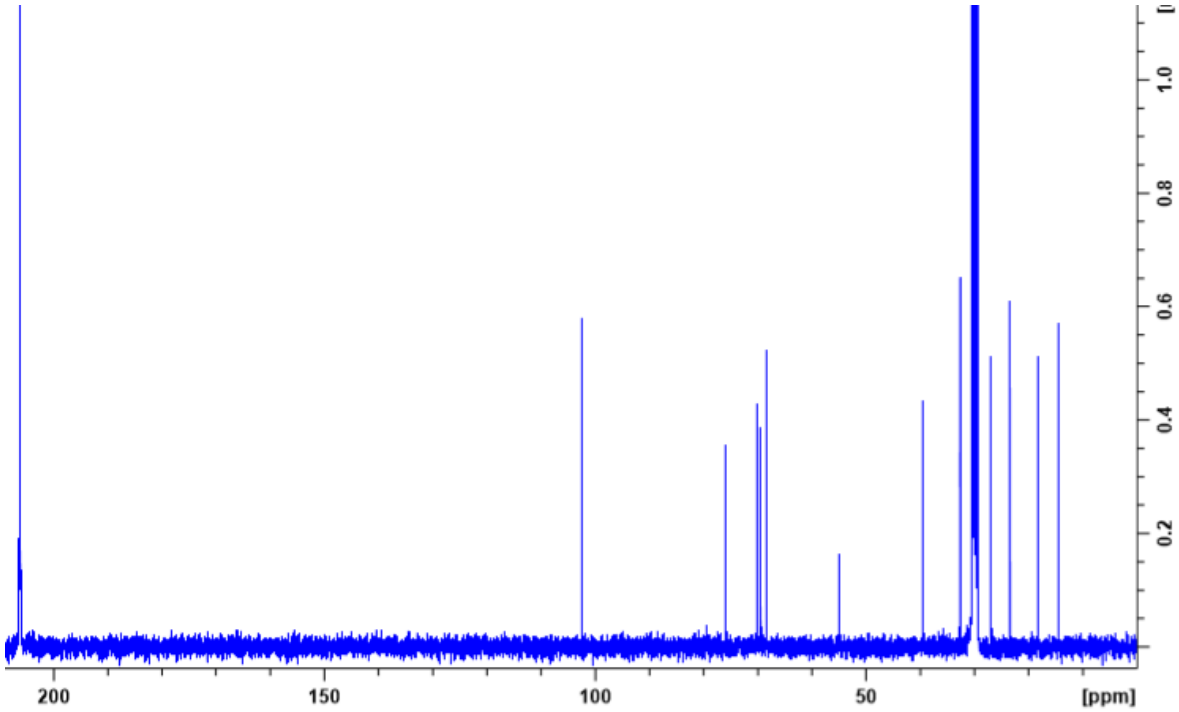

1.2
1.0
0.8
0.6
0.4
0.2
100
80
60
40
20
[ppm]


OCH2(CF2)8CF3
O
AcO
AcO
[rel]
0
2
4
6
8
6
4
2
[ppm]


OCH2(CF2)8CF3
HO
HO
O
5
4
3
2
1
[ppm]
3.0 [rel]
2.5
2.0
1.5
1.0
0.5
-0.0


100
80
60
40
20
[ppm]
2.5
2.0
1.5
1.0
0.5
0.0
-0.5


O(CH2)11CH3
HO
HO
O
I
[rel]
0
2
4
6
8
10
12
5
4
3
2
1
[ppm]


0.2
0.4
0.6
0.8
1.0
200
150
100
50
[ppm]